GROWTH

A JOURNAL FOR STUDIES OF DEVELOPMENT AND INCREASE

VOLUME XXI
PAPERS 509-531

1957

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A JOURNAL FOR STUDIES OF DEVELOPMENT AND INCREASE

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VOLUME XXI, NUMBER 1
PAPERS 509-514

MARCH, 1957 $6.00 A YEAR

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GROWTH

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It is published four times a year during March, June, September and December at $6.00 per volume (outside U. S. and Canada, $6.50). Single numbers $2.00. There will be one volume each year.

All manuscripts and subscriptions should be sent to: The Editors of Growth, Lankenau Hospital Research Institute, Fox Chase, Philadelphia 11, Pa.

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Growth, 1957, **21**, 1-9.

THE EFFECT OF NUTRITIONAL ENVIRONMENT ON BLOOD GLUTATHIONE LEVEL IN BEEF CATTLE[1]

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(Received November 19, 1956)

Rather extensive and conclusive evidence indicates an association between glutathione—GSH—concentration and rate of cell proliferation, protein synthesis and adult size in several animal species. A small negative correlation between blood GSH and subsequent economy of gain, and between blood GSH and heart girth in yearling beef steers was observed by Kidwell et al. (1955).

Kunkel et al. (1954) concluded that blood GSH level of beef cattle 9-13 months of age is an individual characteristic, relatively unaffected by environmental change. It has, on the contrary, been clearly demonstrated that certain environmental factors markedly alter the GSH content of some tissues (Goss and Gregory 1932, 1935 a, b; Gregory and Goss 1933, 1934, 1939; Chanton 1932; Reid, Ward and Salsbury 1948). Kidwell et al. (1955) observed a change in blood GSH of 47 steers from 24.18 $\pm$ 0.79 mg. percent to 31.54 $\pm$ 0.72 mg. percent during an 84-day feeding period. The animals studied by Kidwell et al. were on a low plane of nutrition prior to the first determination, but on a high plane prior to the second, while those studied by Kunkel may have been on a more uniform plane throughout. It was suggested that differences in plane of nutrition might account for the discrepancy.

This paper presents the results of an experiment designed to determine the main effects, and interactions, of diethylstilbestrol, chlortetracycline, and graded levels of animal fat in the ration on blood GSH level in long yearling Hereford steers. Further observations of the relation of GSH to other blood constituents, rate and economy of gain, and body size are also considered.

[1] This work is in cooperation with the Animal and Poultry Husbandry Research Branch, A.R.S., U.S.D.A., under Western Regional Project W-1 on beef cattle breeding research. It was also supported by a grant from the American Cyanamid Company.

Materials and Methods

The experimental animals consisted of 48 steers, predominantly Hereford, ranging in age from twenty-one to twenty-seven months. They were allotted to twelve groups of four steers each on the basis of weight. The groups were randomly assigned to treatments in a 2 x 2 x 3 factorial design. The treatments included two levels of diethylstilbestrol, zero and 10 mg. per steer daily; two levels of chlortetracycline, zero and 70 mg. per steer daily; and three levels of animal fat, zero, 9.8 and 19.6 percent of the concentrate mixture. The twelve concentrate rations are presented in Table 1. Alfalfa hay was fed as a roughage. The feeding, management and analytical procedures described by Kidwell et al. (1955) were employed.

TABLE 1
Physical Composition of Experimental Rations—Percent.

	70 Mg. Chlortetracycline Daily						None					
Ingredient	10 Mg. Diethylstilbestrol Daily			None			10 Mg. Diethylstilbestrol Daily			None		
Fat	0	9.8	19.6	0	9.8	19.6	0	9.8	19.6	0	9.8	19.6
Rolled Barley	24.5	24.5	24.5	24.5	24.5	24.5	24.5	24.5	24.5	24.5	24.5	24.5
Beet Pulp	32.3	32.3	32.3	32.3	32.3	32.3	32.3	32.3	32.3	32.3	32.3	32.3
Ground Milo	41.2	31.4	21.6	41.2	31.4	21.6	41.2	31.4	21.6	41.2	31.4	21.6
Salt	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Bone Meal	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Total	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0

Weights and blood samples were obtained and analyzed initially, after 40 days and 120 days, the completion of the feeding period. The correlation between blood GSH and heart girth (0.42) observed by Kidwell et al. (1955) indicated the desirability of heart girth measurement, but this was precluded by inadequate facilities. The data were amenable to analysis of variance and correlation techniques described by Snedecor (1956).

Results and Discussion

The mean and standard error of each measurement for each period is presented in Table 2. Analysis of variance indicated no differences among treatment groups for any of the observed variables (weight, GSH, Hb, and total cell count) at the start of the experiment.

The data indicate that after 40 days none of the twelve rations resulted in significant differences among any of the four traits measured. At the conclusion of the 120-day feeding period there was evidence of treatment effects on weight, average daily gain, pounds of hay per pound of gain, and pounds of concentrate per pound of gain. The details of this analysis are reported in a companion paper by Bohman, et al. (1957). Dietary treatments did not affect the blood GSH, hemoglobin, or total cell count. It appears reasonable to conclude that under the conditions of this experiment the addition of animal fat, diethylstilbestrol and chlortetracycline to the ration of fattening long yearling steers has no effect on total blood glutathione, hemoglobin, or total cell count, but does influence weight, rate and economy of gain.

The means and standard errors (Table 2) suggest a change in mean values for each trait during the experiment. The change in each measurement for each individual during the first 40 days, last 80 days, and entire 120 days was studied. The mean changes and corresponding 95% confidence intervals are presented in Table 3. Treatment effects on the changes were determined by analysis of variance. There was evidence of treatment effects on change in weight for all three periods and change in blood GSH during the first 40-day period. The

TABLE 2
Means and Standard Errors of Measurements.

Item	Original	40 days	120 days
GSH	33.1 ± .87	29.2 ± .80	39.0 ± 1.46
Total Cell	8.59 ± .12	7.90 ± .15	8.09 ± .42
Hemoglobin	16.6 ± .20	15.5 ± .25	16.9 ± .27
Weight	774 ± 5.9	873 ± 8.5	1047.7 ± 11.1
Average Daily Gain[1]			2.27 ± .08
Lbs. Hay/Lbs. Gain[1]			5.30 ± .16
Lbs. Grain/Lbs. Gain[1]			4.92 ± .15

[1] Determined over entire 120-day period.

TABLE 3
Mean Changes and 95% Confidence Interval During Three Experimental Periods.[1]

	First 40 days	Last 80 days	Entire 120 days
Weight, lbs.	99.0 ± 12.3	175 ± 19.6	274 ± 31.2
Blood GSH, mg. %	—3.9 ± 1.1	9.8 ± 2.5	5.9 ± 2.7
Hemoglobin, gm. %	—1.1 ± .3	1.4 ± 0.6	0.3
Total Cell Count Millions/mm^3	—0.68 ± 0.18	.19	—.49 ± .32

[1] Change not significant where confidence interval omitted.

existence of treatment effects on change in blood GSH and weight during the first 40 days, but not on total blood GSH and weight at the conclusion of the 40-day period is possible because the variance at the end of forty days is composed of the initial variance and the variance of the change, and the initial variance is large in relation to the variance of the change.

The analysis indicated that only one main effect—diethylstilbestrol—and one interaction—chlortetracycline X diethylstilbestrol—were significant. The results are summarized in Table 4. The 24 steers

TABLE 4
The Influence of Diethylstilbestrol and Chlortetracycline on Change in Blood GSH During First 40 Days.

Treatment	Average Change in Blood GSH, Mg. %		
	Chlortetracycline	None	Average
Diethylstilbestrol	—3.0	—7.1	—5.0
None	—5.9	+0.1	—2.9
Average	—4.4	—3.5	—4.0

receiving diethylstilbestrol had an average decrease in blood GSH of 5.0 mg. percent, while those not receiving the hormone decreased an average of 2.9 mg. percent. This difference was significant at the five percent level. The difference in decrease between those receiving chlortetracycline and those not receiving the antibiotic was not significant. The interaction between diethylstilbestrol and chlortetracycline was significant at the one percent level. Apparently diethylstilbestrol and chlortetracycline fed together lowered blood GSH only about half as much as either fed alone. There was no change in blood GSH level of animals receiving neither the hormone nor antibiotic. A physiological interpretation of these results is not immediately apparent. Certainly the treatment effects on blood GSH do not correspond to treatment effects on weight change. It seems clear that the metabolic process or processes involved were of a temporary nature, as the effects were noted only during the first 40 days. Blood GSH increased thereafter. This is in agreement with the previous observations of Kidwell et al. (1955) where neither antibiotic nor hormone was fed.

Significant changes other than those due to treatments were observed for several of the variables (Table 3). The factors responsible for the changes are not evident. Previous work in this laboratory

indicated the chemical analysis to be quite accurate and the results highly repeatable. The pattern of change of blood GSH (a decrease followed by an increase to a higher level than originally) is of interest in view of the findings of Rapkine (1931). Rapkine determined free sulfhydryl at various stages in the first division cycle of *Paracentrotus Lividus*. The free SH decreased during the first stages; then increased to a level above original value. The peak value coincided with egg cleavage.

The correlation of change in GSH, change in hemoglobin, and change in total cell count with weight change were not significantly different from zero. It can be concluded, however, that under the conditions of this experiment blood GSH, hemoglobin and total cell count of fattening cattle are not necessarily stable, but may change rather markedly, contrary to the findings of Kunkle et al. (1954).

The relations among the variables were studied. In those instances where there was no evidence of treatment effects, the entire group of 48 steers was considered as a sample from a single population, and first order product-moment correlations estimated from the entire group. The pertinent estimates are presented in Table 5. The

TABLE 5
Simple Correlation Coefficients.

	Intermediate GSH	Final GSH	Initial Total Cell	Intermediate Total Cell	Initial Hb	Intermediate Hb
Initial GSH	.74**	.41**	.67**	—	.77**	—
Intermediate GSH	—	.53**	—	.64**	—	.67**
Intermediate Hb	—	—	—	—	.66**	—
Final Hb	—	—.09	—	—	.28*	.25
Intermediate Total Cell	—	—	.67**	—	—	—
Final Total Cell	—	—.04	.23	.37*	—	—

* 5% level of significance.
** 1% level of significance.

correlation between GSH and weight was not significantly different from zero for any of the three determinations. This tends to confirm the results of Kidwell et al. (1955). The correlations between determinations at the three different times for GSH, hemoglobin, and total cell count are in agreement with previous estimates. Except for the final determination, the correlations between GSH and hemoglobin

and GSH and total cell are quite high and are in agreement with the previous findings of Kunkle et al. and Kidwell et al. Kidwell et al. noted that these correlations suggested that a large proportion of the blood GSH is in the red blood cells and a smaller proportion in the plasma, and suggested that GSH in the red blood cells might be in depot form and plasma GSH in a dynamic state. Plasma GSH was not found in measurable quantities, even though the plasma was concentrated to one fifth the original volume. The data indicate that virtually all blood GSH is located in the red cells.

In those instances where there was evidence of treatment effects, correlations were determined on a within-group basis. These correlations are presented in Table 6. The observed correlations

TABLE 6
Within Treatment Correlations.

	Initial				
	GSH	Total Cell	Hb	Wt.	Average Daily Gain
#Hay/#Gain	.41**	.61**	.43**	.09	—.91**
#Grain/#Gain	.35*	.42**	.38**	.09	—.85**
Avg. Daily Gain	—.35*	—.39**	—.39**	.01	—

* 5% level of significance.
** 1% level of significance.

between initial GSH and economy of gain (lbs. hay/lbs. gain and lbs. grain/lbs. gain) are in close agreement with those reported previously (Kidwell et al. 1955). The correlation between initial GSH and rate of gain is similar to that observed previously (—.35 vs. —.28) although the previous estimate was just short of significance. It should be noted that since economy of gain is expresseed as pounds of feed per pound of gain, there results an inverse scale of measurement; i.e., the most economical animals consume the least feed per pound of gain. Consequently, low values of blood GSH are associated with most economical, as well as most rapidly gaining, animals. In contrast, previous investigators (see Kidwell et al. 1955) observed a positive association between GSH, rate of cell proliferation, protein synthesis and adult size. These studies, however, involved either the entire animal or growing tissues other than blood. It is possible that blood GSH may be utilized by other tissues. The observations of Mortensen (1953) support this suggestion. The low

levels of blood GSH in the most rapidly and economically gaining animals may reflect a type of depletive state. The correlations between blood GSH at 40 days and previous rate and economy of gain were —.54 and +.41 respectively; and between final blood GSH and previous rate and economy of gain —.80 and +.23 respectively. All except the last are significant at the one percent level. These correlations support the suggestion that low blood GSH levels in the most rapidly and economically gaining animals are indicative of a depletive state. It is even possible that such animals might gain even faster or more economically if given additional GSH or the means of synthesizing it.

The correlation between hemoglobin and rate and economy of gain and between total cell and rate and economy of gain are similar to those observed for GSH and these traits. This is expected since virtually all the blood GSH is found in the red blood cells.

The high correlations between GSH and hemoglobin and total cell count, and the similarity of the correlations between GSH and rate and economy of gain to those between hemoglobin and total cell count with rate and economy of gain suggest that there is no cause and effect relation between GSH, hemoglobin or total cell and rate or economy of gain, but that all three vary as the result of some metabolic process or processes associated with ability to gain rapidly and economically. Partial correlations between GSH and economy of gain and between GSH and rate of gain with hemoglobin and with total cell count held constant were all insignificant, which supports this suggestion.

The ratio of GSH to hemoglobin was determined, and the correlation between this ratio and rate and economy of gain estimated. These correlations were smaller than those between GSH and rate and economy of gain. It is concluded that the relation between ratio of GSH and hemoglobin and rate and economy of gain is too low to be of value in a predicting equation. No relation between initial weight and rate and economy of gain was observed. This was expected, since the group was selected for uniform initial weight. The high relation between rate and economy of gain is in agreement with reports in the literature.

Summary and Conclusions

Forty-eight long yearling Hereford steers were assigned treatments for a 120-day feeding period in a 2 x 2 x 3 factorial experimental design to determine the main effects and interactions of two levels of chlortetracycline, two levels of diethylstilbestrol, and three levels of animal fat on blood GSH, hemoglobin, total cell count, weight, rate and economy of gain. Relations among the variables were also studied.

The analysis indicates that under the conditions of this experiment the following conclusions are warranted:

1. The addition of animal fat, diethylstilbestrol, and chlortetracycline to the ration of fattening long yearling steers has no permanent effect on total blood GSH, hemoglobin, or total cell count, but does influence weight, rate and economy of gain. Diethylstilbestrol and the interaction of diethylstilbestrol and chlortetracycline resulted in a temporary decrease in blood GSH.

2. Blood GSH, hemoglobin, and total cell count of fattening long yearling cattle are not necessarily stable, but may change rather markedly.

3. There is no correlation between blood GSH and weight, but rather high correlations between GSH, hemoglobin and total cell count exist.

4. Small but significant correlations between blood GSH, hemoglobin, and total cell count with rate and economy of gain exist. The evidence suggests that these do not represent primary cause and effect relations, but are possibly the result of more fundamental metabolic processes mediated through the red blood cells.

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VARIATIONS IN GROWTH CHARACTERISTICS OF SEVEN STRAINS OF TETRAHYMENA

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(Received November 21, 1956)

Introduction

Cellular physiologists have recognized for many years the value of protozoan forms in studies of morphology, cytochemistry, and physiological function. More recently much attention has been directed to a study of the various species and strains of the genus *Tetrahymena*. The impressive scope of these studies has been reviewed by Corliss (3). Interest in the form is apparently well justified in that its metabolic activity is, in many respects, notably similar to that of mammalian forms (7, 16).

A large number of strains have been employed in such studies. Corliss (1) has grouped the 26 existing strains into four species and established a basis for their proper identification. Much of the difficulty in study of these particular protozoans has been due to the confusion in nomenclature, since the genus designations *Colpidium, Glaucoma, Leucophrys,* and *Tetrahymena* have been used improperly or interchangeably.

While identification and nomenclature have been clarified, there are still many factors which may be confusing in drawing general conclusions from studies of these ciliates. The strains may vary considerably from one another in cell size (2, 9) but, on the other hand, cell size may depend upon the nutrient employed. Also, the cells may vary quite considerably in size during the several phases of the growth curve (10). Strains also may vary considerably in metabolic activity. Loefer (8) has reported differences in ability of strains to utilize carbohydrates, and there is evidence that amino acid and vitamin requirements are not so firmly fixed as was once believed (5, 15).

If *Tetrahymena* is to be used in studies from which general conclusions may be drawn, it seems essential to note and to appreciate

physiological differences in the strains employed. In order to demonstrate this point, a study has been made of variations in growth characteristics in several strains of the genus. From such an investigation, basic differences may be demonstrated which may have several values. To the protozoologist, they may be a guide in selection of a strain for a particular type of study. To the cellular physiologist, the morphological changes observed during growth, and variations in changes in different strains should be of interest. To the taxonomist, there may be an indication that physiological behavior in a single group may vary even more than do the morphological characters, upon which so much emphasis is frequently placed in classification.

Materials and Methods

For the study, six strains of *Tetrahymena pyriformis* and one of *T. vorax* were used. They were: *T. pyriformis* W, originally collected by C. L. Claff in 1939 at Woods Hole, Massachusetts; *T. pyriformis* L I and L II, both collected by J. B. Loefer in Texas in 1948; *T. pyriformis* HS from a thermal artesian spring in Texas by A. Phelps in 1948-9; *T. pyriformis* CHS collected by E. Chatton at Strasbourg, France, in 1925; *T. pyriformis* E, collected by A. M. Elliott in 1932 in New York City; and *T. vorax* V_1, first collected by D. M. Lilly in Providence, Rhode Island, in 1940. More detailed histories of the strains are available elsewhere (1). The strains were selected because of the variations in original geographic source (except for L I and L II) or original environments.

The strains were maintained as axenic cultures in cotton-plugged Pyrex test tubes containing glucose and Proteose Peptone (Difco) in a special solution of salts (11). The cultures were maintained in darkness at 18°C. and during maintenance, transfers were made with a sterile bacteriological loop to fresh medium every seven days.

Cells for growth tests were obtained from 72-hour stock cultures. Live counts were made of a 0.1 ml. aliquot sample which had been aseptically withdrawn. On the basis of the live count, a sample of the stock culture containing 200 cells was used to inoculate 20 ml. of fresh medium contained in a cotton-plugged Pyrex erlenmeyer flask. Ten such cultures were prepared for each strain and these were incubated in a dark chamber at 24 ± 1°C.

At 12, 24, 48, and 72 hours a 0.1 ml. sample was taken aseptically from each culture. The samples from cultures of a strain were pooled

and the following determinations made. Live cell number was ascertained by direct count, using appropriate dilutions where required. Measurements of cell length and width and nuclear diameter were made of not less than 20 cells fixed in Lugol's solution. An ocular micrometer was employed for the measurements, made at a magnification of 450 X. From such measurements, the cell volume was calculated by use of the formula

$$V = 4/3 \pi A^2 B,$$

where V equals the volume if A is one half of the width and B is one half of the length of the cell. Nuclear volume was calculated by use of the formula for volume of a sphere:

$$V = 4/3 \pi r^3$$

In addition, the cytoplasmic:nuclear ratio was determined and the total protoplasmic volume per ml. of culture calculated (cell volume $\times$ cell number per ml.). Results were analyzed statistically by the "Null hypothesis" and Chi-square determinations in order to support the following statements made in regard to differences in nature of cell morphology or growth activity of the various strains.

Results

Results of all tests are presented in Table I. It is apparent that there is considerable difference in growth characteristics of cells of the seven strains. Most rapid cell division occurs in strain W. Strains E, HS and L II showed slowest cell division, while CHS, L I and V_1 showed intermediate division rates. The slowest continuous rate of growth occurs in strain E, while a prolonged lag phase occurs in strains L I, HS and V_1.

The cell size diminishes as cell division becomes most rapid in strains W, and L I; it remains about the same in strain CHS and increases in strains L II, HS, E and V_1. The size of the nucleus remains the same except in strains L I, L II and E, in which it increases during the growth period of the cultures. The largest cell is that of strain W, while L II and V_1 are smallest; L I, HS, CHS and E are intermediate in size, although the latter increases most notably in size during the prolonged period of culture growth. Additional determinations showed that cultures of strain E continue to grow slowly for an additional 48 hours, although all other strains seem to have approached their peak of culture population at 72 hours.

TABLE I

Growth of *T. pyriformis* W, LI, LII, HS, CHS and E and *T. vorax*, V_1 at 25°±1°C. in a medium containing 1% Proteose Peptone and 1% Glucose as nutrients.

Strain		0 Hr.	12 Hr.	24 Hr.	48 Hr.	72 Hr.
	Cells per ml.	10	580	16,000	168,000	719,700
	Cellular divisions		5.18	4.94	3.32	2.38
W	Cell vol. (μ^3)	15,460	13,920	9,695	11,580	12,600
	Protoplasm (μ1)/ml. culture	0.00015	0.001	0.154	1.95	9.067
	Nuclear volume (μ^3)	560	560	560	560	560
	Cytoplasmic:nuclear ratio	28	25	17	21	23
	Cells per ml.	10	10	152	9,400	198,300
	Cellular divisions		0	3.90	5.93	4.32
LI	Cell vol. (μ^3)	7,890	4,640	4,760	5,550	7,800
	Protoplasm (μ1)/ml. culture	0.000079	0.000046	0.00072	0.052	1.485
	Nuclear volume (μ^3)	110	350	350	350	350
	Cytoplasmic:nuclear ratio	73	13	14	16	21
	Cells per ml.	10	30	126	3,100	29,500
	Cellular divisions		1.50	2.01	4.55	3.18
LII	Cell vol. (μ^3)	3,480	3,400	3,190	4,000	4,200
	Protoplasm (μ1)/ml. culture	0.000035	0.0001	0.0004	0.012	0.124
	Nuclear volume (μ^3)	110	350	350	350	350
	Cytoplasmic:nuclear ratio	32	10	9	11	12
	Cells per ml.	10	30	52	1,150	22,300
	Cellular divisions		1.50	2.30	4.39	4.10
	Cell vol. (μ^3)	5,720	6,380	9,450	7,780	6,380
HS	Protoplasm (μ1)/ml. culture	0.000057	0.000191	0.00049	0.009	0.425
	Nuclear volume (μ^3)	350	350	350	350	350
	Cytoplasmic:nuclear ratio	16	18	27	22	18
	Cells per ml.	10	51	529	21,500	323,700
	Cellular divisions		2.27	3.30	5.27	3.94
CHS	Cell vol. (μ^3)	4,520	4,910	4,890	4,960	6,430
	Protoplasm (μ1)/ml. culture	0.000045	0.00025	0.0026	0.107	2.08
	Nuclear volume (μ^3)	350	350	350	350	350
	Cytoplasmic:nuclear ratio	13	14	14	14	18
	Cells per ml.	10	53	186	2,000	21,100
	Cellular divisions		2.33	1.76	3.36	1.30
E	Cell vol. (μ^3)	7,850	11,050	13,100	14,950	12,610
	Protoplasm (μ1)/ml. culture	0.000079	0.00059	0.0024	0.03	0.066
	Nuclear volume (μ^3)	350	560	840	840	890
	Cytoplasmic:nuclear ratio	22	20	16	18	14
	Cells per ml.	10	10	105	7,900	103,100
	Cellular divisions		0	5.66	6.17	3.67
V_1	Cell vol. (μ^3)	3,170	5,040	4,930	5,150	4,900
	Protoplasm (μ1)/ml. culture	0.000032	0.000050	0.00052	0.041	0.506
	Nuclear volume (μ^3)	350	350	350	350	350
	Cytoplasmic:nuclear ratio	9	14	14	16	14

The period of maximum division rate appears most quickly (within the first 12 hours) in strain W, the strain showing best growth. Other strains took longer to attain the maximum division rate. The division rate for strain E remains generally low throughout the period of the tests, indicating a slow rate of continuous growth.

Discussion

A study of growth processes within cultures such as those used in this study may consider three factors: (1) change in the size of individual cells, (2) increase in total number of living cells present, and (3) increase in total protoplasmic volume. Too frequently cell number is the only basis upon which results of a growth study are evaluated. However, it has been demonstrated that the size of the cells may vary at different phases of the culture growth cycle. Ormsbee (10), using strain H of *Tetrahymena pyriformis,* showed that in the initial phases of growth the individual cells became shorter, then later increased in length; this apparently was due to differences in the rates of cellular assimilation and of cellular divisions. He also determined cell count and the total dry weight of cellular material present. However, cell volume was not calculated. Zeuthen (17) correlated growth of cell isolates (*T. pyriformis* W and GL) with respiratory activity. He noted that growth was rapid immediately after cell division. Then followed a period of slow growth previous to the next division. Scherbaum and Zeuthen (14) furnish evidence that excessively increased cell size (artificially induced by temperature variation so that division is arrested) eventually leads to an excessively rapid rate of cell division. Thus, growth and division must eventually return toward a proper balance.

Protoplasmic content in a culture has been estimated by the photometric method (4). However, it must be emphasized that differences in intracellular content may cause considerable difference in light absorption by two culture samples even though the cell number and size may be similar. Thus, the value of this method is dependent upon appreciation of its possible limitations. It would seem that the laborious method of cell counting and measurement finally gives the most complete information regarding growth in a mass culture population.

While others have reported a decrease in cell size early in the culture growth cycle, such is not the case for all strains reported upon in this study. Only in W, L I and L II do cells become smaller as

the maximum division rate is approached. Cells of strains CHS and V_1 remain at about the same size during the entire period of the culture growth while cells of strain HS may increase initially in size, then become smaller, and in strain E the cells continuously increase in size during the observed period of the culture growth. In the latter instance, this may be related to the extremely slow rate of continuous increase in the population density. Division processes do not exceed assimilation in the cell of strain E.

In the studies described in this paper, it is noted that there may be considerable difference in average cell size when comparing the seven strains used. When the growth tests were started, the average cell volume of cells from stocks grown for 72 hours at 18°C. was in the order W> L I> E> HS> CHS> L II> V_1. After a 72-hour growth period at 25°C. the order was W> E> L I> HS> CHS> V_1> L II. Actually, some of the changes were quite considerable, as the tabulated data indicates; e.g., the cell of strain E increased to nearly the same size as that of W, although it was considerably smaller at the beginning of the test. Apparently the difference in temperature for growth was an important factor; strain E, especially, grows to a much greater size at 25°C. than it does at 18°C. There are some differences in cell size observed here from that reported by other authors. Loefer (9) studied growth of several strains of Tetrahymena, growing them in a salts solution with added Bacto Casitone (2 per cent) and Bacto Yeast Extract (0.25 per cent). Five of the strains which he used were the same as those used in this report. The cells, after 48 hours culture growth at 27.5°C., were, in the order of decreasing size, E> W> L II> L I> HS. Corliss (2) cultured the available strains of Tetrahymena on "bacterized" hay infusion at room temperature and made measurements of cells from 5- to 7-day-old cultures. He found that the seven strains reported on in this paper would be, in order of decreasing cell size, E> W> HS> L II> V_1> L I> CHS. It appears that in all three studies cells of strain W and E are the largest. Discrepancies in comparison of other strains may be due to differences in temperature of incubation, nature of nutrients and age of culture. The fact that temperature for culture growth influences cell size has been mentioned earlier in this paper.

One of the aspects of cellular growth and division of concern to physiologists is the possible nature of conditions conducive to the initiation of cellular division. An interesting theory, first presented by

Hertwig (6), proposed that the size of the nucleus controls the size of the cell. A certain amount of nuclear substance would preside over the accumulation of cytoplasm to a point, then the nucleus was forced to duplicate itself and cytoplasmic fission followed. Popoff (12) measured the cell and nuclear size and calculated their volumes in *Frontonia leucas*. He found the cell·nuclear ratio reached its highest value prior to cell division and was followed by a rapid increase in nuclear volume immediately preceding fission. He reported that a similar situation obtains in *Paramecium caudatum* (13). This apparently does not apply for all Tetrahymena strains studied here. In strains W, HS, E and V_1 the cytoplasmic:nuclear ratio is higher before or during the period of most rapid division, then levels off or falls during the next 12 or 24 hours. The ratio remains constant in strain CHS and in strains L I and L II it falls off to a lower level before most rapid division occurs. The similarity of growth characteristics in the latter two strains is of particular interest in view of the common original point of isolation. It would appear that if the nucleus is the most important structure in initiation of cellular division, then its size alone is not the primary factor for all strains of Tetrahymena.

From results of this study, there are indications that certain strains of Tetrahymena may lend themselves to particular types of investigation. Strain W might be best for metabolic studies, since the cell is large and divides rapidly, thus furnishing the greatest protoplasmic yield per culture volume. Actually, this strain has enjoyed extensive use in such areas. Because of the large cell size and slow, prolonged growth, strain E might be best suited to the careful analysis of changing conditions during the growth of the culture. The possible nature of nuclear influence upon cellular fission might best be ascertained by comparison of strains L I and L II with W and V_1. It should be noted that in regard to characteristics of growth which have been considered here, *T. vorax* V_1 shares more features with *T. pyriformis* than do strains within the species.

Conclusions

1. Growth characteristics have been determined for six strains of *Tetrahymena pyriformis* (W, L I, L II, HS, CHS, E) and one strain of *T. vorax* (V_1).

2. While the temperature at which growth occurs has some influ-

ence on cell size, generally cells of strains W and E are of largest size; strains L II and V_1 are smallest.

3. Most rapid cell division occurs in strain W, while strains E, HS and L II show slowest rates of cell division.

4. Cells become smaller during the period of most rapid division in strains W, L I and L II, remain about the same size in strain CHS, and increase in size in strains HS, E and V_1 as most rapid division occurs.

5. Nuclear size remains about the same through the growth period except in strains L I, L II and E, in which case it increases.

6. The cytoplasmic:nuclear ratio is high before or during most rapid division in strains W, HS, E and V_1, remains nearly constant in strain CHS, and falls to a lower level prior to the period of most rapid cell division in strains L I and L II.

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VARIATIONS IN THE INHIBITORY EFFECT OF POTASSIUM CYANIDE UPON GROWTH AND RESPIRATION IN SEVEN STRAINS OF TETRAHYMENA[1]

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Received November 21, 1956

Introduction

Studies of cyanide effects upon metabolism have shown that *Tetrahymena pyriformis* W may be inhibited in its growth and respiratory activity in relatively high concentrations of KCN (e.g., 10^{-3} M) while the same processes might actually be stimulated if KCN is present in lower concentrations (e.g. 10^{-9} M) (16). In addition, the same strain of cells become adapted, by prolonged growth in the presence of KCN, so that partial resistance against the growth and respiratory inhibition may be gained (14, 15). Many other strains of *Tetrahymena* are available (4) and have been employed in a great number of physiological investigations (5). However, it has been noted that there may be considerable variation in certain physiological activities. For example, different strains show variability in utilization of a large number of carbohydrates (10) and certain strains may alter their metabolism in regard to amino acid requirements (8) and vitamin synthesis (21). There is considerable difference in normal growth responses in some strains of *Tetrahymena* (11).

In view of the variability in metabolism and because of the possibility that there may be individual variations of strains in response to the presence of cyanide, a study of the effect of cyanide upon growth and respiration of several strains of *Tetrahymena* was undertaken. Such a study might be informative in two regards: (1) it would serve to illustrate the similarity or diversity of strains in an aspect of their physiological behavior and (2) it might eventually

[1] This investigation was supported in part by a research grant E-850 from the National Microbiological Institute, of the National Institutes of Health, Public Health Service.

lead to more information regarding possible mechanisms in resistance to cyanide inhibition.

With these points in mind a study of the effect of high concentrations of KCN upon growth and respiration in seven strains of *Tetrahymena* has been undertaken.

Materials and Methods

For these studies, seven strains of *Tetrahymena pyriformis* (W, L I, L II, HS, CHS, E) and one of *T. vorax* (V_1) were employed. Complete histories of the strains have been considered by Corliss (5). The cells were maintained in stock culture at 25°C. in a medium and by a method previously described (18).

For growth tests, cells were used from a 72-hour stock culture. The culture population density was determined from an aseptically-withdrawn aliquot sample using the Klett-Summerson colorimeter equipped with a blue filter (7). Assuming that a reading of 60 is indicative of a culture population of approximately 100,000 cells of *T. pyriformis* W per ml. (14) a proper volume of the stock culture was transferred to 180 ml. of culture medium, contained in a 250 ml. Pyrex erlenmeyer flask, so that a final concentration of 100 cells per ml. would be obtained. In a similar manner, cell suspensions of the other six strains were prepared to furnish a protoplasmic content per unit volume similar to that for Strain W. Using a sterile replicating pipette (Clay-Adams Aupette), 20 cultures were prepared from the suspension of each strain. These cultures were of 10 ml. each, contained in rubber-stoppered 15 x 125 mm. Pyrex test tubes. During dispensing of test tube cultures, constantly equal cell suspension was maintained in the erlenmeyer flask by a gently rotating Teflon-coated magnetic bar activated by a magnetic stirrer. After preparation of the test-tube cultures, sterile KCN was added to each of 10 cultures of a strain to give a final concentration of 10^{-3} M KCN. No change in pH of the medium occurred as a result of cyanide addition. The ten remaining cultures of a strain contained no KCN and served for control cultures.

A second method of culture was employed in which duplicate cultures (70 ml. of culture solution in rubber-stoppered 125 ml. Pyrex erlenmeyer flasks) were inoculated, each with 0.4 ml. of a 72-hour stock culture. To one of the flasks KCN was added at 10^{-3} M con-

centration; the second flask served as control. All cultures were incubated at 25°C. for 72 hours after which the culture density was determined with the Klett-Summerson colorimeter.

Respiration tests were conducted using the direct method of Warburg (22). Flasks of approximately 5 ml. capacity were used. Into the main compartment was introduced 1.8 ml. of a 72-hour culture of the strain being studied. In all cases the cell density was adjusted to give a Klett-Summerson colorimeter reading of 60. Adjustment of the culture density was accomplished by concentration of cells by gentle centrifugation or by dilution with supernatant fluid from a centrifuged portion of the culture. In this way, the cell:medium and protoplasmic: inhibitor (KCN) ratios were maintained as nearly equal as possible. The importance of this has been considered as of possible significance for best comparison of results (6). One half of all flasks contained no KCN; the others contained KCN at 10^{-3} or 5 x 10^{-3} M concentration. The center well of control flasks contained 0.2 ml. of N KOH solution, while in test flasks 0.2 ml. of a balanced KOH-KCN solution (9) absorbed CO_2 while preventing cyanide loss, by distillation, from the main compartment. Into the side arm of all flasks, 0.3 ml. of 4 N H_2SO_4 was introduced.

Flasks were equilibrated for 15 minutes at 25°C., then respiration tests conducted for one hour, readings being taken at 15 minute intervals.

In order that the Q_{O_2} could be calculated, cells in aliquot portions of the final cell suspensions were washed by centrifugation in a salt solution (18). After three such washings, a sample was dried to constant weight at 100°C. and correction made for the calculated amount of salt contained. Thus the dry weight of cellular material represented by cells in a flask could be determined, and the Q_{O_2} determined.

Results

While growth was slightly higher in flask cultures than in cultures in test tubes, the variation was generally the same for all strains. Also, the degree of inhibition by 10^{-3} M KCN was similar regardless of the method of culture. Therefore, the average of all determinations is given in Table I. The data indicate that strains W and E are the most sensitive to cyanide inhibition of growth processes.

In Table II the results of respiration studies are given. It is inter-

esting to note that the same two strains, W and E, are most sensitive to cyanide inhibition of respiration and growth. Further, since 10^{-3} M KCN was only very slightly inhibitory to other strains, more tests were conducted on these strains exposed to 5 x 10^{-3} M KCN. Even at this higher concentration strain L I is still notably resistant to respiratory inhibition by cyanide.

TABLE I.

The Effect of 10^{-3} M KCN Upon Growth in Seven Strains of Tetrahymena When Grown for 72 Hours at 25° C. in a Medium Containing Proteose Peptone and Glucose as Nutrients.

Strain	Klett-Summerson Reading		
	Control (0-KCN)	Test (10^{-3} M KCN)	Test as per cent of control
W	60.5	4.0	6
L I	45.5	7.0	15
L II	29.5	6.0	20
HS	20.0	3.5	18
CHS	28.0	3.5	13
E	9.0	0.5	6
V_1	33.5	4.0	12

TABLE II

The Effect of 10^{-3} M and 5 x 10^{-3} M KCN Upon Respiration by Seven Strains of Tetrahymena as Determined by the Direct Method of Warburg at 25° C.

Strain	M KCN	Number of tests	Q_{O_2}	Test as per cent of control
W	0	16	202	
	10^{-3}	16	51	25
L I	0	16	178	
	10^{-3}	8	173	97
	5 x 10^{-3}	8	166	92
L II	0	16	142	
	10^{-3}	8	140	98
	5 x 10^{-3}	8	73	51
HS	0	16	140	
	10^{-3}	8	146	104
	5 x 10^{-3}	8	123	88
CHS	0	16	212	
	10^{-3}	8	191	90
	5 x 10^{-3}	8	185	88
E	0	16	119	
	10^{-3}	16	24	20
V_1	0	16	170	
	10^{-3}	8	187	104
	5 x 10^{-3}	8	119	70

Discussion

Normal growth as observed in these tests occurs more rapidly in strains W and L I, while E grows most slowly. This substantiates other work with these strains. It appears, therefore, that comparative growth rates are established within the strains and not altered by the nature of the culture container or, within limitations observed, by the size of the original inoculum.

It is interesting to note that the strains which were most sensitive to cyanide inhibition of growth (W and E) were also those most sensitive to respiratory inhibition. This indicates the probable involvement of similar metabolism for both growth and respiratory activity.

While Commoner (3) points to the probability that the more active processes involved in respiration are most notably sensitive to cyanide, this claim is not substantiated by results here presented. In these studies strain W displays the most rapid growth and is second only to CHS in respiratory activity. As might be expected, this cell is highly sensitive to KCN. On the other hand, strain E is remarkably slow in both its growth and respiratory activities. It is also highly sensitive to cyanide. While L I is highly resistant to cyanide inhibition of respiration and moderately resistant to growth inhibition, it grows and respires rather rapidly. These findings indicate that cyanide sensitivity or resistance may be determined by more than merely the rate at which metabolism occurs. More complicated factors, probably interacting with one another, probably are involved.

In view of strain variations observed here, it is not surprising that so much controversy existed in earlier studies regarding the effect of cyanide upon respiration. While respiration in *Paramecium* has been shown to be cyanide sensitive (1, 2, 17), Lund (12) reported no inhibition in that species. Although Lund's results may have been due to loss of cyanide from test solutions, strain variation might also have been involved. Respiration in *Glaucoma pyriformis* in peptone solution has been reported to be inhibited temporarily by KCN, later returning to normal (13). This may be indicative either of a loss of cyanide from the medium or of an adaptive mechanism. Closely related *Colpidium* may be insensitive to cyanide (19, 20). Although the variety of results has been largely due to differences in technique and physiological status of the cells used, nevertheless, the possibility of strain variations should not be disregarded in any of these cases.

The results of this study should furnish information of value for continued consideration of factors involved in cyanide resistance, whether it be a natural one, inherent in strains, or an acquired one resulting from previous cyanide exposure. To this end, comparison of metabolism in strains L I and W, the latter as a "normal" or "adapted" clonal culture should be especially suitable.

Conclusions

1. Growth and respiratory tests were conducted on six strains of *Tetrahymena pyriformis* (W, L I, L II, HS, CHS, E) and one strain of *T. vorax* (V_1).

2. Most rapid growth occurs in strains W and L I. Growth is slowest in strain E.

3. Respiratory rate is highest in strains W, CHS and L I, and lowest in strain E.

4. Strains W and E are most highly sensitive to cyanide inhibition of growth and respiration. Strain L I is highly resistant to cyanide inhibition of respiration and moderately resistant against growth inhibition by cyanide.

5. The possible value of the normal variation in cyanide sensitivity to studies upon acquired resistance is discussed.

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SOMITE GENESIS IN THE CHICK
I. PARTIAL CHARACTERIZATION OF STIMULATORY FACTORS IN EGG WHITE[1]

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(Received November 29, 1956)

INTRODUCTION

Although the somites of vertebrate animals are about the first embryonic structures to appear, little is actually known about the biochemical and physiological events accompanying their formation. The early study of Hyman (1927) has indicated that their respiration is not particularly sensitive to inhibitors, and, in fact, they are not centers of high respiratory activity (Moog, 1943). Buno (1951) has indicated that there is no strong reaction with nitroprusside in early chick somites, although more recently Amprino (1955) has found that somite mesoderm of 4- and 5-day embryos has a high uptake rate of S^{35}. The discrepancy in the results of these two workers may be because of the (1) difference in age of the chicks used, (2) undetectability of —SH groups, particularly in some proteins (Barron, 1951) by nitroprusside, or (3) the use of sulfur primarily for other purposes than the formation of thiols and disulfide linkages.

Waddington and Deuchar (1953) found a positive correlation between developing somite depth and length in *Triturus* embryos. The possibility of segmentation being brought about by the diffusion process was ruled out, because this would lead to a negative relationship in the dimensions of these structures.

Recently Spratt (1955) has reported the presence of a pair of somite-organizing centers located slightly posteriolaterally to the node of definitive-streak chick blastoderms. The physiological relationship between these centers and the somites which develop later is not de-

[1] This research has been supported in part by a grant G-1406 from the National Science Foundation.

scribed, although their presence is essential for normal meristic segmentation.

With this very sketchy information as a basis, a study of somite genesis, with its nutritional, histological and serological facets has been initiated. It is the purpose of the present communication to describe the results obtained from a study of the effects of albumen fractions on the development of somites in the early chick. Further papers will be concerned with the roles of sulfur, yolk fractions and intracellular proteins in this event.

Materials and Methods

While most of the eggs used in this study were from White Leghorn chickens, a few were from Rhode Island Red stock. Fresh fertile eggs were incubated at 37.8°C. in a forced-draft incubator for 21 hours, yielding blastoderms that were rather uniformly at the definitive-streak stage (stage 4 of Hamburger and Hamilton, 1951). Only those embryos at this (DPS) stage will be considered in this report. The observations arising from the use of older explants will be given in a later communication. The blastoderms were excised from the yolk and trimmed under chick Ringer's solution, and explanted onto the various media. Camera lucida diagrams were made at this time. The embryos were cultured in a forced-draft tissue incubator at 37.6°C. for 22 to 23 hours. At this time another diagram of them was made with the help of the camera lucida. Most blastoderms were fixed with Gerhardt's fixative and permanently mounted on slides *in-toto* for further inspection. Carazzi's hematoxylin was used as a stain. Somite counts were made from the prepared whole mounts for greater accuracy. Over one thousand embryos were used in the course of this investigation.

Preparation of Media

Basic components. The Ringer's solution was the same as that described by Spratt (1947), except that the NaCl content was reduced to 123 millimolar. This concentration was found to be more compatible to embryonic chick tissues by Howard (1953) than that previously used, and it has recently been adopted by Spratt (1955). In all media, with the exception of that containing whole egg white, the final agar concentration was 0.625% (W/V), while in the exceptional case it was 0.50%. Phenol red was used in the media as an inside pH in-

dicator. Microbial infection was controlled by the use of buffered crystalline penicillin G potassium (Lederle, 250 units/ml. medium) and streptomycin and crystalline dihydrostreptomycin sulfates (Lederle, 0.1 mg./ml. medium) in the media. Contamination in the petri dishes was prevented by soaking the cotton rings on which the watch glasses lay with 0.5% sodium sulfadiazine. Phosphate and bicarbonate buffers (Fraser, 1954, 1956a) were used in all except the whole albumen medium.

Albumen dialysate. One hundred and fifty ml. of fresh whole egg white were dialized against a total of 75 ml. of Ringer's in cylinders whose diameters were only slightly greater than that of the expanded casings. Dialysis was carried out for 3 days in the refrigerator at 5°C. The dialysate was then collected and stored in the refrigerator until used. A concentrated sample of the dialysate gave a negative nitroprusside test, even after reduction with KCN, indicative of the absence of both thiols and disulfide linkages.

Dialized albumen. Seventy-five ml. of fresh egg white were dialized for 3 days against repeated changes of chick Ringer's solution in the cold. The dializing casings containing the albumen were suspended in 10-liter flasks. The large-molecule content of the egg white was then collected for use.

Ovalbumin. The preparation of crystalline ovalbumin has been described previously (Fraser, 1956b). This protein was isolated from whole egg albumen by repeated crystallization with sodium sulfate.

Boiled dialysate. Fifty ml. of freshly prepared albumen dialysate were refluxed mildly for 10 minutes.

Condensed dialysate. Fifty ml. of the dialysate were condensed to half volume by overnight distillation *in-vacuo* at 37.8°C. Air was gently bubbled through the preparation during this time through a finely-drawn capillary tube. At termination the fluid was decanted from the heavy froth.

Alkali fraction of albumen dialysate. To 40 ml. of albumen dialysate was added sufficient 1 N NaOH to bring the pH of the preparation to 11.5. After a few hours the precipitate was brought down in a clinical centrifuge. Both the base-insoluble and soluble fractions were neutralized with HCl and rid of excess salt by solvent extraction as described by Harris (1953). The volumes were slowly reduced to zero under a heat lamp while the preparations were mildly agitated by jets of air directed at them. The salty residues were then treated

with methanol and acetic acid (2 vols. former : 1 vol. latter) and centrifuged. The relative salt-free supernatants were extracted repeatedly with redistilled ethyl ether until all traces of the methanol and acid had been removed. The aqueous phases were again dried under lamp and air. The volume of the base-insoluble fraction was brought to 4 ml., and that of the base-soluble fraction to 20 ml.

Ether partition of albumen dialysate. Fifty ml. of the dialysate were shaken on five occasions with 15 ml. of redistilled ethyl ether. The pooled ether phases were evaporated *in-vacuo* in a cold finger at 45° C. to near dryness. Five ml. of Ringer's were then added and the residual ether driven off with continued distillation. The aqueous phase was treated similarly for the removal of all traces of ether.

Pepsin hydrolysate of dialized albumen. To 50 ml. of dialized albumen was added one-half gram of pepsin (Nutritional Biochemicals, 1 : 10,000). The pH was reduced to 2.7 with HCl and the mixture incubated at 37.6° C. for 2 days. The resultant digest was dialized against 20 ml. of chick Ringer's solution for two days in the cold. The dialysate was neutralized with half-normal NaOH and rid of excess salt as outlined above. The final volume was brought to 10 ml. with Ringer's. A sample of this preparation gave a strong nitroprusside test after reduction with KCN.

Acetone fractionation of albumen dialysate. Preliminary empirical tests had revealed that glutamic acid, the least aqueous-soluble amino acid found free in the dialysate, could be crystallized in a mixture of 2 parts redistilled acetone : 8 parts glass-distilled water. Both glucose and alanine (the most aqueous-soluble amino acid in the dialysate) required a 1 part acetone : 9 parts water for crystallization. On the basis of these observations the following procedure was adopted. Thirty ml. of fresh dialysate were concentrated under the heat lamp, while a jet of air was passed over the fluid. When the volume had been reduced to 5 ml., 20 ml. of redistilled acetone were added and the mixture was left in the refrigerator overnight. On the following day the crystalline portion was centrifuged down. Five ml. of Ringer's solution was added to the residue bringing it into solution, and the acetone was distilled off *in-vacuo* in a cold finger at 60° C. A sample of this gave a negative test with Benedict's reagent, indicating the absence of the glucose. All traces of acetone were distilled from the supernate in a similar manner. The final volume was adjusted to

5 ml. A sample of this fraction gave a very strongly positive Benedict's test.

Nitroprusside Test

The test used in the detection of sulfhydryl groups and disulfide linkages in the albumen dialysate and pepsin hydrolysate was based somewhat on the histochemical method described by Bourne (1953). To 1 ml. of the test solutions were added 2 drops of 5% acetic acid. This was followed by the addition of 5 drops of 5% sodium nitroprusside in saturated ammonium sulfate and 2 drops of concentrated ammonia. The rapid development of an ephemeral purple color indicates the presence of free —SH groups. This, however, did not occur in either of the two materials tested. For a qualitative estimation of oxidized sufhydryls, 3 drops of 10% KCN were added to the tubes. This material reduces each disulfide linkage to 2 —SH groups, which then react with the nitroprusside.

Chromatographic Analysis of Albumen Dialysate

Fifteen ml. of the dialysate were evaporated to give a volume of approximately 2 ml. Several drops of the concentrate were applied to 18 by 22-inch Whatman paper No. 1. Descending chromatography was employed with water-saturated phenol as the first solvent. The second dimension was brought about by a second run using lutidine —collidine—water (3 : 1 : 1 by volumes) as the solvent. This procedure has been outlined by Dent (1948). The finished papers were dried, sprayed with a 0.1% solution of ninhydrin in 95% ethanol, and dried in an oven for several minutes. The results of this chromatographic analysis of the free amino acids in egg white are shown in Figure 1. Six amino acids were readily recognized by their positions: aspartic acid, glutamic acid, lysine, alanine, valine and leucine(s). The leucines cannot be separated with certainty by this method.

Contents of Media

Table 1 indicates the composition of the various media used in the investigation on somite formation. The compounds in the column at the left are the test materials in each case. Each value given represents the number of milliliters of the particular substance used. Gelation of the media with a total volume of 4 ml. occurred in depression slides held in petri dishes.

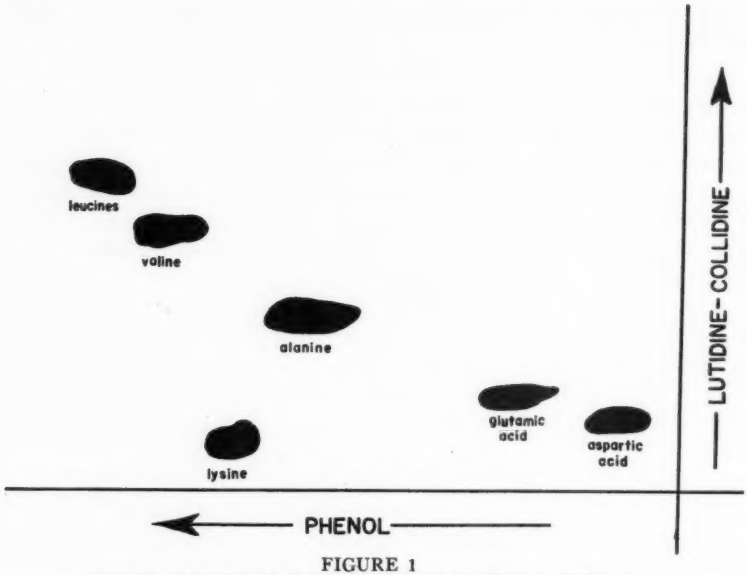


FIGURE 1
Localization of ninhydrin-positive spots of albumen dialysate.

RESULTS

The results can most readily be shown in table form. The average number of pairs of somites developing in chick blastoderms on each medium is given in Table 2 along with the standard error for those groups in which somites formed. All explants were cultured for 22 to 23 hours.

As has been pointed out previously (Fraser, 1954), there is no appreciable development on a non-nutrient agar preparation. This is reflected in a failure of somites to form and the node to regress, along with degenerative changes, particularly at the region of the node.

It may be seen that the average number of somite pairs developing in DPS blastoderms cultured on an albumen medium is 5.6. This is followed by an average of 4.7 observed in explants cultured on the dializable fraction of whole albumen (Figure 2). The proximity of these two values would suggest that the essential nutrilites for somite development in the young chick are, for the most part, in the form of small molecules. This contention is supported by the observation

TABLE 1
Components of Media Used in Chick Somite Formation.

Test Material	Ml. test cmpd.	Ringer	Phenol red	Pen.-Strep.	Phos. Buff.	Bicarb. Buff.	Total Volume
None	—	33	2	2	2	1	40
Albumen	20[1]	26	2	2			50
Alb. Dialysate	20	13	2	2	2	1	40
Dialyzed Alb.	20[1]	26	2	2			50
Ovalbumin (0.5%)	20	23	2	2	2	1	50
Glucose (1%)	8	25	2	2	2	1	40
Gluc. + Dial. Alb.	28[2]	18	2	2			50
Boiled Dialysate	20	13	2	2	2	1	40
Condensed Dial.	15	18	2	2	2	1	40
Boil., Cond. Dial.	15	18	2	2	2	1	40
Base-insol. Fraction	2	1.3	0.2	0.2	0.2	0.1	4
Base-sol. Fraction	20	13	2	2	2	1	40
Ether-sol. Fraction	2	1.3	0.2	0.2	0.2	0.1	4
Saline-sol. Fraction	20	13	2	2	2	1	40
Free Amino Acids	24[3]	9	2	2	2	1	40
Gluc. + Glut. Acid	12[4]	21	2	2	2	1	40
Gluc. + Glutamine	12	21	2	2	2	1	40
Gluc. + Alanine	12	21	2	2	2	1	40
Gluc. + Alan. + H_2S	12	21[5]	2	2	2	1	40
Glucose + H_2S	8	26[5]	2	2	2	1	40
Pepsin Hydrol.	2	1.3	0.2	0.2	0.2	0.1	4
Gluc. + Pep. Hydrol.	2.8[6]	0.5	0.2	0.2	0.2	0.1	4
Acetone Ppt. Dial.	2	1.3	0.2	0.2	0.2	0.1	4
Gluc. + Acetone Ppt.	2.8[6]	0.5	0.2	0.2	0.2	0.1	4
Supernate from Acetone Ppn.	2	1.3	0.2	0.2	0.2	0.1	4

[1] Twenty-five ml. of whole albumen were mixed thoroughly with 50 ml. of chick Ringer's. Twenty ml. of froth-free liquid were decanted for use.

[2] Eight ml. of 1% glucose plus 20 ml. dialized albumen.

[3] Four ml. each of the 6 detected amino acids in concentration of 100 mg.%. These were DL aspartic acid, DL leucine, DL valine, DL lysine HCl, and L glutamic acid.

[4] Eight ml. of glucose (1%) plus 4 ml. of glutamic acid (100 mg.%). The same proportions were used in the next three media.

[5] H_2S, prepared in a generator, was bubbled through an aeration stone into chick Ringer's solution. Time: 10 minutes.

[6] Four-fifths ml. of glucose (1%) plus 2 ml. of the other component.

that somite genesis is not existent on media in which the principal ingredients are ovalbumin alone or the entire large-molecule moiety of egg white (Figure 4). Even when augmented with 200 mg.% glucose, the protein fraction of albumen is quite ineffective. There does appear to be some somite-forming capacity in the protein fraction, however, becoming apparent on its hydrolysis, when supported by glucose as an energy source.

The results reveal that with gentle refluxing or aeration in the process of condensation, albumen dialysate loses some of its ability to

TABLE 2
Somite Development on Various Media

Medium	No. Embryos	Avg. No. Pairs Somites ± St. Error	Medium	No. Embryos	Avg. No. Pairs Somites ± St. Error
Saline-agar	20	0	Saline-sol. Fraction	21	1.8 ± 0.5
Albumen	32	5.6 ± 0.3	Free Amino Acids	20	1.9 ± 0.5
Albumen Dialysate	34	4.7 ± 0.5	Gluc. + Glutamic Acid	15	0
Dialized Albumen	30	0	Glucose + Glutamine	15	0
Ovalbumin	18	0	Glucose + Alanine	23	2.0 ± 0.3
Glucose	109	0	Gluc. + Alanine + H_2S	20	3.4 ± 0.6
Gluc. + Dial. Alb.	20	0	Glucose + H_2S	36	1.7 ± 0.2
Boiled Dialysate	16	2.3 ± 0.5	Pepsin Hydrolysate	18	0
Condensed Dialysate	22	2.4 ± 0.4	Gluc. + Pepsin Hydrol.	17	1.8 ± 0.5
Boil. & Cond. Dial.	17	2.0 ± 0.3	Acetone Ppt. of Dial.	19	0
Base-insol. Fraction	21	0	Gluc. + Acetone Ppt.	22	0
Base-soluble Fraction	16	1.9 ± 0.3	Supernate of Acet. Ppn.	17	2.1 ± 0.6
Ether-sol. Fraction	14	0			

promote the development of somites. A combination of the two procedures does not further reduce this capacity, however, suggesting that the same labile factor is affected by both treatments. It is immediately apparent that all of the important nutrilites are not precipitated by an alkali. It is also suggestive that although no active factor is present in the ether-soluble fraction of the dialysate, such treatment has impaired the somite-forming factors in the aqueous phase similar to that seen in the alkali-soluble fraction. There is the possibility that one active component may be present in both the alkali-soluble fraction and the ether phase, but is inactivated by treatment with these organic solvents. The same effect is brought about by acetone precipitation. Again the activity of one fraction showing activity is curtailed, and to approximately the same degree.

It may also be noticed that blastoderms cultured on a medium containing the amino acids found free in egg white have an average number of somite pairs of 1.9. One of the amino acids, glutamic acid, has been shown to be of considerable importance in intermediary metabolism in recent years. Both it and its analogous glutamine, however, are of no value in the formation of somites in the chick, when they are offered in this manner. Corollary tests using the other amino acids detected, with the exception of alanine, when used in conjunction with glucose, were also ineffective. It can be seen that somites do form to a limited extent on an agar gel containing alanine (Figure 3). The results obtained when hydrogen sulfide, with and without alanine, was included in the culture medium are interesting, because here one can see a cumulative effect.

Two other observations not concerned directly with somite formation will be given briefly at this time. In all explanted embryos there is a certain tendency for development to digress to some degree from that seen in *in-ovo* embryos. However, when certain features appear in explants on a particular medium in sufficient numbers, one is aroused to the suspicion that the medium is likely to be somewhat responsible for the anomaly seen. The conditions described in the following cases were observed in the majority of explants on each medium.

Typically, blastoderms cultured on dialized albumen show a marked suppression of the mid-region, accompanying the failure of somites to form. These embryos have both a head and tail frequently well elevated above a flat sheet of tissue between them. In several cases no

FIGURES 2-5

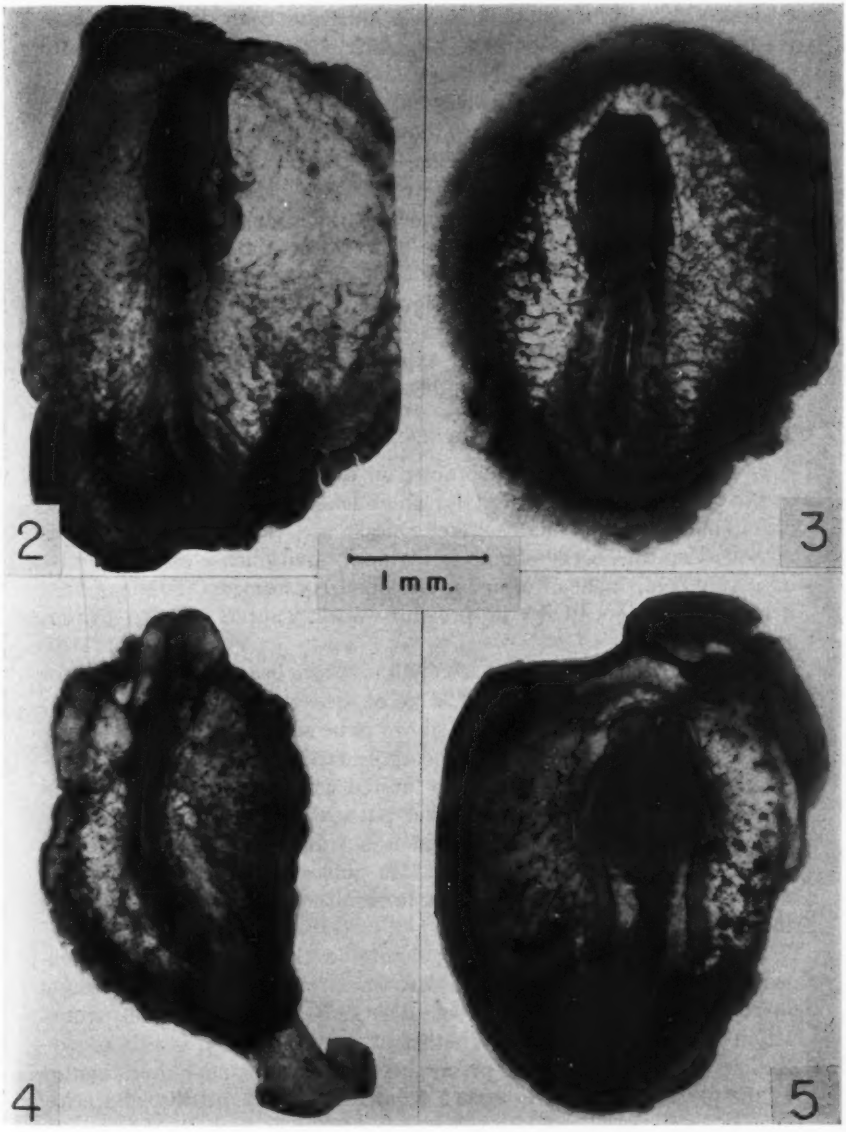
Details of chick blastoderms cultured on various media. See text for further details. Figures 2, 3 and 4 were explanted at DPS stage, Figure 5 at head-fold stage.

FIGURE 2
Dializable fraction of whole albumen.

FIGURE 3
DL alanine supplemented with glucose.

FIGURE 4
Dialized albumen.

FIGURE 5
Pepsin hydrolysate of dialized albumen.


2
3
1 mm.
4
5

evidence of a brain could be seen within the head. Some of these features are seen in Figure 4. On such a medium containing only large molecules differentiation has definitely been impaired selectively in regions. Cell proliferation had not been affected, however, because explants on this medium were about the only ones with large outgrowths of cells from their margins.

Embryos cultured on pepsin hydrolysate of dialized albumen fortified with 200 mg.% glucose showed a definite anomalous condition in the vascular system. In these the heart forms anterior to the head. To the heart leads a pair of very long and large vitelline veins not at all typical of other explants (Figure 5). The embryo shown in this figure has only a fore- and hind-brain, with mid-brain lacking. This condition, seen in a number of explants on various media, suggests the discontinuous nature of the development of the brain.

Discussion

In examining the results obtained in the present study, one is led to the general conclusion that for chick embryos cultured *in-vitro* the requirements for normal somite genesis are rather complex. It is obvious that an energy source is required, and glucose as a naturally occurring component of albumen fills this requirement. This has been demonstrated repeatedly in previous work (Spratt, 1948; Fraser, 1954).

But above this need for a carbohydrate there is a further demand for at least three other components. One of these appears to be the amino acid alanine, because it has been shown to be somewhat effective in promoting somite formation when incorporated into the culture medium. Further, it could account for the limited activity of preparations resulting from boiling, aeration, ether partition and alkali and acetone treatment, because this amino acid is not destroyed in any sense by these procedures. Alanine, along with glucose is not precipitated by 80% acetone, and it is the supernate resulting from this fractionation that shows some activity.

How alanine acts in promoting somite development is partially conjecture. The results will reveal that when it is added to the medium in conjunction with hydrogen sulfide, there is an appreciable stimulation to somite formation. Whether or not the embryo is able to synthesize cysteine from these precursors is a matter under investigation at the present time. It is known from recent work in this laboratory

that the number of pairs of somites which form in explants on agar containing cysteine is considerably larger than that in embryos on whole albumen. The results of work dealing with the sulfhydryl effect on somite formation will be published shortly. In this regard it should be mentioned that both alanine and cysteine have recently been shown to stimulate morphogenesis in the early development of the fish, *Fundulus* (Trinkaus and Drake, 1956). The possibility does exist, however, that the sulfide may be active in its own right, acting as a reducing agent for intracellular enzymes (Barron, 1951).

The nature of the other stimulatory component of small molecular size (if only one exists) in egg white is not as clearly defined. It would seem that it is quite labile in nature, losing activity on boiling and aeration as well as treatment with ether and acetone, or accompanying manipulations. Such adverse effects may be manifested by the destruction of the molecule as a whole or to its active groups. Further work is required before a more complete characterization of this factor can be presented.

There is some evidence that the large-molecule fraction of whole albumen also contributes to the formation of somites. This is in agreement with the observation of Taylor and Schechtman (1949) that there is a certain need by embryonic chicks for the protein moiety of egg yolk for their normal growth. A survey of Table 2 will reveal that whole albumen is slightly better than its dialysate for meristic segmentation. Further, blastoderms on a gel containing the digest of dialized albumen (with added glucose) developed somites to a limited degree. It is impossible to give the nature of the active component with assurance, but other observations have indicated that it may well be a sulfhydryl-containing compound. Cystine is found in abundance in the proteins of albumen (Block and Bolling, 1951), although lacking in its dialysate. In fact, the dialysate gives a negative nitroprusside test even after reduction with KCN. The peptic digest of dialized albumen, however, gives a strong reaction. It has already been mentioned previously that cysteine, a sulfhydryl-containing amino acid, strongly stimulates the development of somites in explants. These observations lead one to suspect that the active component in the protein fraction may be a sulfur-bearing compound. Certainly the enzymes are present in the embryo for protein hydrolysis. The observation of Buno (1951) that sulfhydryls are in greatest concentration in the area apaca of the blastoderm, a region which

is in contact with the sulfur-containing proteins of the yolk, has been confirmed in this laboratory.

Summary

1. An assay of the stimulatory effect of various fractions of egg albumen on somite formation in the early explanted chick embryo has been undertaken.

2. Albumen dialysate is only slightly less active than whole egg white in this regard. Somite genesis is impaired to about the same degree in explants cultured on media containing the dialysate which has been either boiled, or aerated thoroughly or treated with a strong base.

3. The free amino acids found in egg white have a slight stimulatory effect on somite formation. It has been shown that one of the occurring amino acids, alanine, alone could duplicate these results. Its activity is augmented upon the further addition of hydrogen sulfide to the medium.

4. There is some development of somites in blastoderms cultured on agar gel containing the pepsin hydrolysate of dialized albumen, when accompanied by glucose.

5. The conclusion is reached that there are at least four components in whole egg albumen which act synergically in the normal development of somites in the early chick explant. These are believed to be: (1) glucose, (2) a labile factor whose nature is as yet obscure, (3) alanine, whose activity may lie in its transformation to cysteine, and (4) a component of the protein moiety, which may be a sulfur-containing compound.

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ADAPTATION TO THE EFFECTS OF AMINOACETONITRILE UPON SKELETAL STRUCTURES[1]

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It has long been known that rats fed on diets containing large amounts of *lathyrus odoratus* seeds develop bone deformities. These are due to the formation of numerous exostoses, especially at muscle-insertion sites, and to irregular proliferation of cartilage, particularly in the rapidly growing epiphyseal plates (3, 6, 7, 15). The active principle was first extracted in impure form (5) and, subsequently, it has been possible to demonstrate that β(γ-L-glutamilamino)propionitrile is present in the seeds of *lathyrus odoratus* and duplicates the effects of this plant upon the skeleton (1, 2, 8). Although the presence of aminoacetonitrile (AAN) has not been demonstrated in *lathyrus odoratus* seeds, it is even more effective than β(γ-L-glutamilamino) propionitrile (16) in this respect.

The literature on lathyrism has been the subject of an excellent review (14), so that we need not discuss it here in detail. Suffice it merely to add that, more recently, we have learned that the response of bone, either to diets containing *lathyrus odoratus* seeds or to subcutaneously administered AAN, can be influenced at will: cortisol, ACTH or thyroxine inhibit, while STH or partial hepatectomy augment, the bone changes characteristic of experimental lathyrism (9-13).

It has been our impression that growing rats, chronically treated with AAN, become gradually less sensitive to this drug. However, our observations did not prove the acquistion of resistance to this substance because it is well known that the skeleton of young rats is more sensitive to AAN that that of older animals. In view of the many similarities between experimental bone-lathyrism and certain naturally occurring diseases of man (idiopathic scoliosis, Paget's disease, Legg-Perthes' disease, degenerative arthritis), it seemed of pos-

[1] This work was supported by the Defense Research Board of Canada, Grant No. 9310-35, Project D50-93-10-35.

sible practical importance to establish whether the skeleton can be rendered resistant to the actions of a drug such as AAN. It is the object of this communication to report upon experiments which show that, within certain limits, this is feasible. At the same time, we wish to call attention to certain basophilic and aldehyde-fuchsin-positive corpuscles which appear with great regularity in the bones of AAN-treated rats.

Materials and Techniques

Twenty female Sprague-Dawley rats, having an initial body-weight of 101 gm. (range: 93—105 gm.), were subdivided into two equal groups. Group I acted as non-pretreated controls, while Group II was pretreated with AAN (in the form of aminoacetonitrile hydrosulphate) as follows: 1st—13th day, 6 mg.; 14th—31st day, 10 mg.; 32nd—36th day, rest; 37th—45th day, 10 mg.; 46th—50th day, rest; 51st—61st day, 10 mg. Beginning on the 62nd day, the animals of both groups were given 20 mg. of AAN daily for an additional 15 days and were then killed. The daily dose of AAN was always administered subcutaneously in two equal doses (at 9.00 A.M. and 4.00 P.M.) in 0.2 ml. of water. After autopsy, the whole skeleton was macroscopically examined and the distal end of the femur fixed and decalcified in Susa solution for subsequent histologic examination. After being embedded in paraffin, the sections were stained either with hematoxylin-eosin or with Gomori's aldehyde-fuchsin technique (4).

Results

Even mere macroscopic inspection of the skeleton sufficed to show that the bones of the non-pretreated animals in Group I were more severely deformed than those of the pretreated rats in Group II. The exostoses at the muscle-insertion sites, the diffuse periostal new-bone formation, as well as the proliferative changes in the growth cartilage plates, were all less intense in the rats which had been adapted by pretreatment during the first 61 days of the experiment (Fig. 1).

Histologic examination revealed changes characteristic of experimental lathyrism in the bones and cartilage plates of all animals, but again, the intensity of the lesions was much more pronounced in the non-pretreated group.

In order to obtain an objective indicator of bone proliferation, the narrowest diameter of the shaft was measured with calipers on each

of the femora. The mean of these measurements was 5 mm. (range: 4.5—5.5 mm.) in the non-pretreated, and 3.2 mm. (range: 3.0—3.5 mm.) in the pretreated rats.

These observations indicate that, if treatment with AAN is started with comparatively low doses and is then gradually raised (with occasional rest periods to permit recovery), the effect of the drug is not cumulative. On the contrary, some degree of adaptation is possible, since the rats given only 15 days of treatment with 20 mg. of AAN per day were more severely affected than those which, prior to this same treatment, had already been given ascending doses of the same drug during a little over two months.

In our earlier work on the bone-lathyrism caused by the feeding of *lathyrus odoratus* seeds (11), it was noticed, incidentally, that newly-formed bone spicules often contain dust-like, small, basophilic granules. These have now been subjected to a more careful study, because they appear to be rather characteristic of this particular skeletal lesion.

In the present experiment, basophilic corpuscles were constantly present in all animals of both groups. They were found in the matrix of newly-formed bone spicules, as well as in that of cartilaginous areas in the process of bony metaplasia. During AAN intoxication, cartilage tissue tends to develop in the periosteum, particularly as muscle-insertion sites, and is frequently transformed into bone by direct metaplasia. In such areas, the basophilic corpuscles were especially common, but we did not find them in cartilage showing no tendency towards ossification, for example, in the cartilaginous covering of joint surfaces and in the uncalcified layers of the epiphyseal plates.

The size of the corpuscles varied between just visible dust-like particles and globules of about 1 μ in diameter. On hematoxylin-eosin preparations, the corpuscles stain dark blue, but their outlines are not sharply delimited. Usually, these structures are round, and, in the larger globules, the center is comparatively light, as if it were hollow. Occasionally, several corpuscles appear to coalesce into mulberry-like structures, and sometimes they are arranged in chains which seem to follow the course of the bone canaliculi into which the osteocytes extend their processes. The corpuscles also exhibit a definite tendency to aggregate around the capsules of osteocytes or of chondrocytes, when the latter are in the process of metaplasia into osteocytes (Fig. 2). On sections stained with Gomori's aldehyde-fuchsin tech-

FIGURE 1

Macroscopic aspect of a typical femur of a non-pretreated (top) and of a pretreated rat given the same amount of AAN during the last 15 days of the experiment. The bony exostoses and the periosteal bone formation are manifestly more evident in the rat which had received no pretreatment.

FIGURE 2

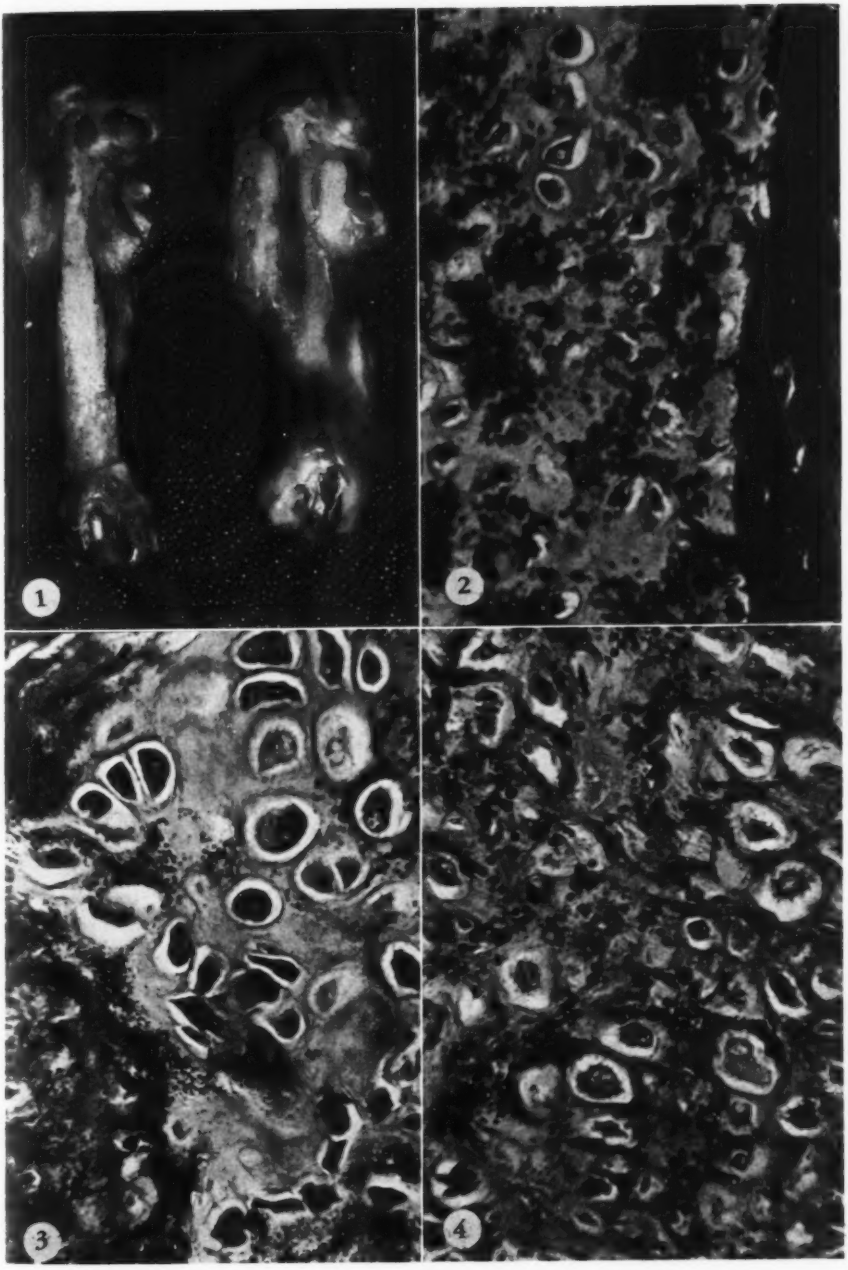
Darkly staining globules in the matrix of newly-formed periosteal bone. An older, deeply staining, bone spicule is seen near the lower edge of the field (hematoxylin-eosin X 400).

FIGURE 3

Cartilaginous spicule in the process of ossification. Numerous globules of various sizes are seen, especially around the capsules of the ossifying cartilage cells and in the line of demarcation between the darker staining bone spicule (lower part of the field) and the lighter staining cartilage (aldehyde-fuchsin X 400).

FIGURE 4

Cartlaginous tissue near a muscle-insertion site in the process of ossification. In this region, the globules are particularly coarse, and many of them tend to aggregate into mulberry-like structures (aldehyde-fuchsin X 400).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nique, (especially after mordanting with 1% oxalic acid or 0.8% periodic acid for 5 minutes), these bodies are much more clearly demarcated, in the form of dark-red granules (Figs. 3 and 4).

In order to ascertain the specificity of this change, we have examined a number of sections through the bones of normal rats and of animals in which skeletal lesions had been produced by trauma or overdosage with parathyroid hormone or dihydrotachysterol. In none of these tissues was there any comparable development of basophilic corpuscles, although occasionally, faintly staining granules of a somewhat similar type may appear in small bone spicules of rats not treated with AAN, especially in the course of callus-formation. However, this is quite exceptional, and if an occasional group of granules is formed, its outlines are usually indistinct, and the basophilic material tends to permeate the matrix diffusely, instead of accumulating in corpuscular form. A study of human bones affected by various disease processes also failed to reveal similar structures, except in small areas of a humerus affected by congenital syphilitic osteochondritis.* It appears, therefore, that within the bone material examined, *the massive appearance of basophilic, aldehyde-fuchsin-positive globules is a characteristic feature of experimental lathyrism.* The possible significance of these corpuscles in the pathogenesis of lathyrism remains to be elucidated but, judging from their tinctorial properties, they may reflect abnormal calcium deposition.

Summary

Experiments on rats indicate that pretreatment with gradually increasing doses of aminoacetonitrile (AAN) imparts a certain degree of resistance to subsequent intoxication with large doses of the same compound.

Certain basophilic and aldehyde-fuchsin-tingible globules are described in the intercellular substance of bone and in the ossifying cartilage matrix. These corpuscles appear to be rather characteristic of experimental bone-lathyrism.

* Apparently, these globules are closely related to, if not identical with, the corpuscles of incipient calcification, that tend to occur especially in embryonic bone at tendon-insertion sites. These bodies have been referred to as "Kalkspuren" by F. Weidenreich, in: Handbuch der mikroskopischen Anatomie des Menschen. [Ed.: W. v. Möllendorf, Springer, Berlin, 1930], Vol. II/2, p. 476.

Acknowledgment

The author gratefully acknowledges generous supplies of AAN ("Amino-acetonitrile Hydrogen Sulphate") from Abbott Laboratories.

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EFFECTS OF NUCLEOPROTEIN FRACTIONS FROM ADULT AND JUVENILE TISSUES ON GROWTH OF CHICK FIBROBLASTS IN PLASMA CULTURES[1]

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(Received January 16, 1957)

INTRODUCTION

Current investigations of cell nutrition *in vitro* have emphasized the use of completely defined media (Evans, Bryant, McQuilkin, Fioramonti, Sanford, Westfall and Earle, 1956; Healy, Fisher, and Parker, 1955; Morgan, Campbell and Morton, 1955; Waymouth, 1955; White, 1955), or of a synthetic medium supplemented by minimal amounts of dialyzed serum (Eagle, 1955a). These techniques have made possible the indefinite cultivation of Strain L mouse cells in the absence of protein (Healy et al., 1955; Evans et al., 1956) as well as the specific determination of amino acid and other nutritional requirements for certain cell types (Eagle, 1955b-e).

While protein-free synthetic media will contribute increasingly to the field of cell nutrition it may be well to emphasize the possibility of alternative or shifting patterns of nutrition for cells in protein-rich and protein-poor media respectively. Thus the He La strain of human uterine carcinoma cells will proliferate actively in a synthetic medium supplemented only with small amounts of serum protein (Eagle, 1955c). But if these cells are grown instead in 20 percent chick embryo extract and 40 percent horse serum, less than half of the cellular protein is derived from free amino acids, the balance coming apparently from the external proteins of the medium (Westfall, Peppers, and Earle, 1955). Similarly, chick fibroblasts will survive for many months in protein-free nutrients (Morgan, Morton, and Parker, 1950; Morgan et al., 1955). In serum-embryo extract, however, chick fibroblasts appear to synthesize cellular protein predominantly from pre-

[1] Aided by grants from the Atomic Energy Commission, Division of Biology and Medicine, and from the American Cancer Society.

cursors above the amino acid level, utilizing large amounts of protein as well as free amino acids from the external medium (Francis and Winnick, 1953; Lu and Winnick, 1955).

These observations underline the desirability of continued nutritional studies with protein-containing media as well as with fully defined synthetic nutrients, and of a continued analytical search for significant growth factors from natural sources, especially growth-stimulating proteins. The existence of nucleoprotein growth factors in chick embryos was suggested by the classic studies of Carrel, Fischer, and other contemporary workers (see summary in Fischer, 1946), but the inadequacy of early methods for separation did not permit a precise definition of the active factors. More recently Kutsky (1953) has developed procedures for the selective separation of a nucleoprotein fraction from chick embryos by precipitation with streptomycin and synergistic effects of this fraction with dialysate factors have been described (Harris and Kutsky, 1954).

Little as yet is known concerning the growth-promoting effects of nucleoprotein fractions obtained from adult tissues, although an extensive series of investigations has been published by Doljanski and his collaborators on the effects of saline extracts or acetone precipitates obtained from various organs of adult chickens. The initial papers in this series (Doljanski, Hoffman, and Tenenbaum, 1939; Hoffman and Doljanski, 1939; Hoffman, Tenenbaum and Doljanski, 1939, 1940) together with the parallel experiments of Trowell and Willmer (1939) showed that stimulation of mitotic activity and increase in surface area with chick fibroblasts could be obtained by saline extracts from a variety of organs from adult chickens. Extracts from adult heart and brain were comparable in potency to embryo extract and permitted the cultivation of heart fibroblasts for six months to a year without decline in growth rates (Doljanski and Hoffman, 1943). These extracts were partially heat stable, contained both dialyzable and nondialyzable growth factors, and could be purified partially by precipitation with alcohol and treatment by lipid solvents (Werner and Doljanski, 1942; Doljanski and Doljanski, 1944; Doljanski and Werner, 1945; Margoliash, Tenenbaum and Doljanski, 1948). Subsequently Margoliash used acetone to dessicate fresh organs from adult chickens and also to fractionate saline extracts from these dried tissues (Margoliash, 1947, 1950; Margoliash and Doljanski, 1950).

The experiments described above suggest the presence of protein

growth factors in extracts from adult chicken tissues. In the present investigation we have explored this possibility more specifically by the isolation of nucleoprotein fractions from adult organs using appropriate modifications of the streptomycin procedure mentioned previously. We have also compared the activity of extracts obtained from adult, juvenile and embryonic sources and with acetone fractions of adult tissues as prepared by Margoliash. Details will be outlined in subsequent sections.

Isolation Procedures

The preparation of nucleoprotein fractions (NPF) from adult and juvenile organs was similar in principle to the isolation of NPF from chick embryos (Kutsky, 1953). Organs were obtained from freshly killed adult (2-3 years) chickens, or from newly hatched (one day) chicks, chilled rapidly to 0°C. and maintained at this temperature through subsequent stages with sterile precautions. Each batch of organs (100-200 gm.) was processed in a Waring blender with two volumes of M/8 potassium borate, pH 7.2, until a smooth homogenate was obtained. The homogenate was then dialyzed overnight with stirring against 100 volumes of the borate buffer, and the debris centrifuged off at 20,000 x g for 30 minutes in a Servall refrigerated centrifuge. The supernatant was made M/100 in streptomycin sulfate, and after standing one hour was dialyzed overnight against borate. The precipitate which had formed was centrifuged down at 20,000 x g for 15 minutes, resuspended in ten volumes of M/3 sodium chloride and dialyzed against borate as before. A second precipitation was carried out by adding streptomycin sulfate to the dialyzed material to M/100. After one hour the precipitate was centrifuged off and resuspended in five volumes of M sodium chloride, buffered to 7.2 with M/100 phosphate. The resulting mixture was dialyzed with stirring for 36-48 hours against 100 volumes of M sodium chloride, and ultracentrifuged for one hour at 105,000 x g. At this stage the lipid cap was drawn off and discarded, and the clear middle fraction dialyzed 36 hours with stirring against 100 volumes of Gey's solution. The resulting solution, after a final clarification in the ultracentrifuge, was used for experiments as the final nucleoprotein fraction (NPF).

The fractionation of adult organs with acetone followed the procedures described by Margoliash (1947, 1950). Fresh adult organs were dessicated with acetone and subsequently extracted for 16 hours

with Gey's solution at 0°C. The resulting extract was precipitated by the addition of acetone at 0°C. to 75 percent final volume. After drying over $CaCl_2$ in vacuum a brownish powder was obtained from each of the adult organs. For experimental studies this powder was extracted with Gey's solution and the considerable insoluble residues centrifuged off. The final solutions were clear and reddish brown. These will be referred to as the acetone-insoluble fractions (AIF).

Characterization of Fractions

Dry weights were obtained on each of the final assay fractions. Aliquots were dried overnight in a vacuum oven at 102°C. and the resulting figures corrected for salt content. These values were used in establishing appropriate concentrations for assay cultures, and in comparing the relative yields of the various fractions studied (Table 1). The data obtained indicate that the total NPF obtained

TABLE 1
Comparative Yields of Assay Fractions, Expressed in Milligrams Dry Weight Per Gram Wet Weight Original Organs.

Organ	NPF,* adult organs	NPF,* one-day chick organs	NPF,* whole 12-day embryos	AIF,** adult organs
Heart	0.32	0.61	—	13.1
Liver	2.88	1.31	—	36.1
Spleen	3.61	—	—	21.5
Brain	2.12	1.40	—	18.1
Embryo	—	—	2.41	—

* Nucleoprotein fraction.
** Acetone insoluble fraction.

per gram fresh tissue does not differ significantly from embryo to adult. Lower yields of NPF from adult and juvenile heart are probably the result of difficulties in the initial extraction procedure. Acetone insoluble materials (AIF) were obtained in large amounts from adult tissues but the assay data, to be presented later, show that the specific activity of AI fractions is lower than the corresponding NP fractions.

Ultraviolet absorption spectra were obtained for all fractions, using a Beckman recording spectrophotometer. Liver, heart and spleen NPF, whether from adult or juvenile organs, gave spectra generally similar to that of embryonic NPF (Kutsky, 1953) with a maximum at 260 mμ and a minimum at 240 mμ. The spectrum from juvenile or adult brain NPF showed a flattened maximum at 267 mμ and a minimum at

247 mμ. The ultraviolet absorption spectrum of each of the AI fractions resembled the spectrum for brain NPF but with a further displacement of the maximum to about 273 mμ.

Total nucleic acids in each assay fraction were estimated from the ultraviolet absorption spectrum by comparing absorption at 260 mμ with standard values obtained with known concentrations of Schwartz ribonucleic acid. The data obtained are summarized in Table 2. Consistently high values were obtained for adult liver and spleen. The nucleic acid content of the acetone insoluble fractions was well below the levels found in NP fractions. Preliminary analyses were also performed for desoxyribonucleic acid in some fractions, using the method of Ceriotti (1952). The data obtained indicated that DNA accounted for one-tenth or less of the estimated total nucleic acids.

TABLE 2
Estimated Percentage Total Nucleic Acid in Assay Fractions.

Organ	NPF,* adult organs	NPF,* one-day chick organs	NPF,* whole 12-day embryos	AIF,** adult organs
Heart	9.1	13.2	—	1.6
Liver	18.1	8.6	—	7.7
Spleen	20.3	—	—	3.5
Brain	3.9	4.3	—	2.7
Embryo	—	—	5.1	—

* Nucleoprotein fraction.
** Acetone insoluble fraction.

Methods of Culture

The fractions under study were assayed in Carrel D-3.5 flasks, using a plasma coagulum and fluid supernatant. Explants in various experiments were obtained from the frontal bone of 13-day chick embryos, and from heart or skeletal muscle of the leg in 14-day embryos. The tissue fragments were trimmed to 1.5 mm. in diameter, using a grid, and were inoculated singly into Carrel flasks containing 0.5 ml. chicken plasma. The medium was clotted by the addition of 1.0 ml. Gey's solution containing a minimal amount of embryo extract (0.01 ml. EE_{50} per flask). Subsequently 1.0 ml. supernatant was added containing 20 percent horse serum (Difco) and 80 percent Gey's solution. Test fractions were furnished in Gey's solution for incorporation in the saline fraction of supernatant and clotting fluid. Appropriate levels of concentration were established, based on previous determina-

tion of dry weight for each fraction. The completed medium also contained 100 units/ml. sodium penicillin G (Merck), 50 units/ml. dihydrostreptomycin (Pfizer) and 0.002 percent phenol red. The cultures were incubated at 38°C. Fluid changes were made at four and seven days and the assays terminated at 10 days. Changes in surface area were recorded by tracing the cultures at 15X and a projectoscope. Areal increases (final area minus initial area) were determined with a planimeter and the data treated statistically. Routine pH measurements were made on the supernatant fluids at seven days, using a Beckman pH meter, model G. Lactic acid was also determined in the seven-day supernatants by the method of Barker and Summerson (1941).

Experimental Studies

Growth stimulation with nucleoprotein fractions from adult chicken organs

The results of a number of experiments showed clearly that NP fractions from all adult organs tested (brain, liver, spleen, heart) stimulated active proliferation and outgrowth in cultures of chick fibroblasts. The pattern of effects was essentially the same in all cases. Cultures without added NPF showed a narrow zone of outgrowth after a few days, with the peripheral cells granulated, heterotypic in form, and without conspicuous orientation. Cultures containing NPF at 0.4 mg/ml or more displayed a large expanding outgrowth zone, clear, conspicuously uniform cells which were long and slender and marked radial orientation. These changes were most clearly seen in cultures supplemented with brain NPF and can be illustrated by the photographs shown in Fig. 1. Similar changes were observed with spleen, heart and liver NPF but the outgrowths were more vacuolated and the cell picture slightly more variable.

The effect of NP fractions from adult organs on cell morphology and colonial pattern was accompanied by an increase in surface area of assay cultures over a ten-day period, as well as the production of lactic acid in the culture medium. Figure 2 illustrates these changes with adult brain NPF and shows the correlated rise in surface area and glycolysis as the level of NPF is increased in the assay medium.

To see whether the stimulating effect of adult NP fractions was selective for particular cell types, comparative assays were made with cultures of frontal bone, heart, and skeletal muscle. For convenience

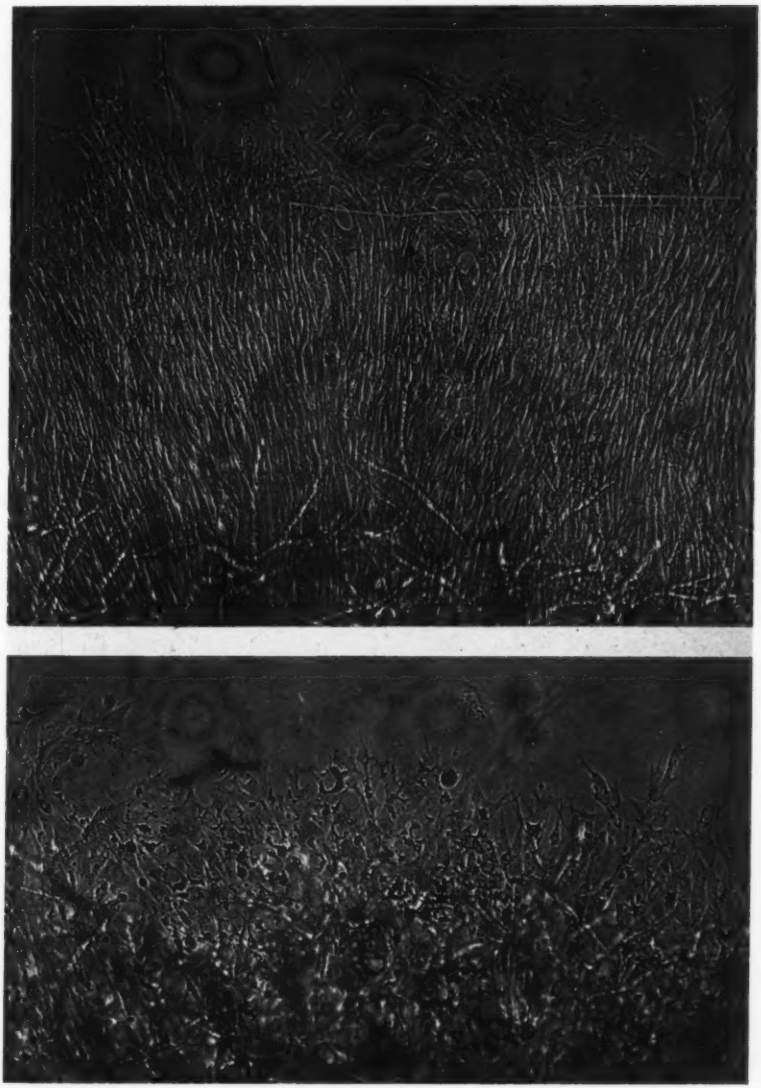

FIGURE 1

Effect of nucleoprotein fraction from adult chicken brain on four-day cultures of skeletal muscle fibroblasts. *Upper:* Marginal area of culture supplemented with brain NPF at 0.4 mg./ml. *Lower:* unsupplemented control culture. (X132).

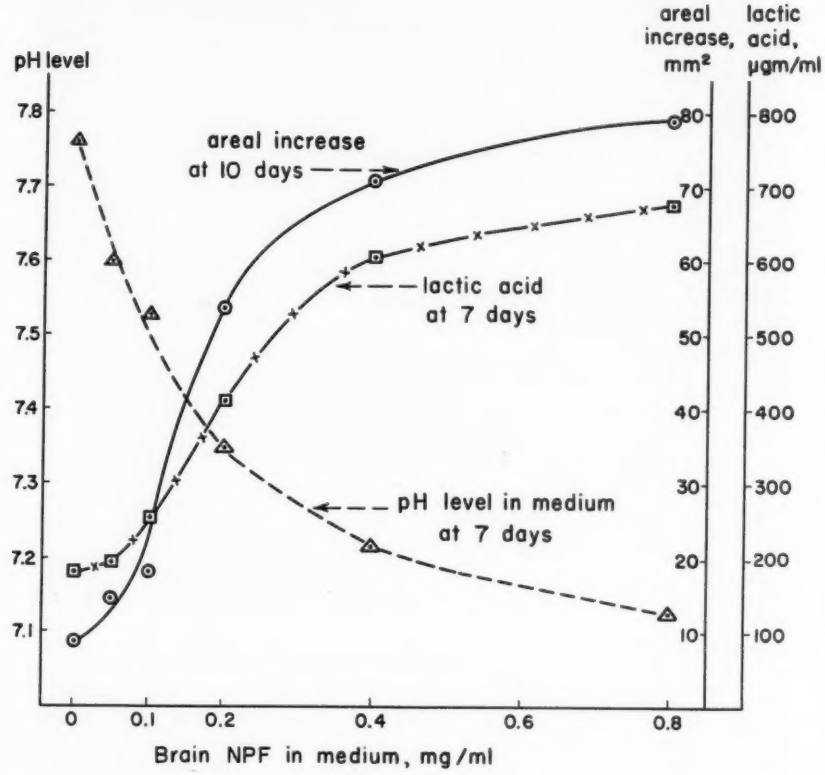

FIGURE 2
Acid production and areal increase in cultures of chick frontal bone.

most of these comparative assays were carried out with adult brain NPF. Figure 3 shows the results of this study, using increase in surface area as a basis for comparison. In cultures of skeletal muscle there was a sharp initial rise to a clear-cut plateau as the concentration of NPF is increased. With explants of frontal bone the approach to a limiting value was more gradual. Cultures of heart showed a lesser response but did not reach a plateau state within the range of NPF concentrations used.

In additional investigations the specific activity of different adult

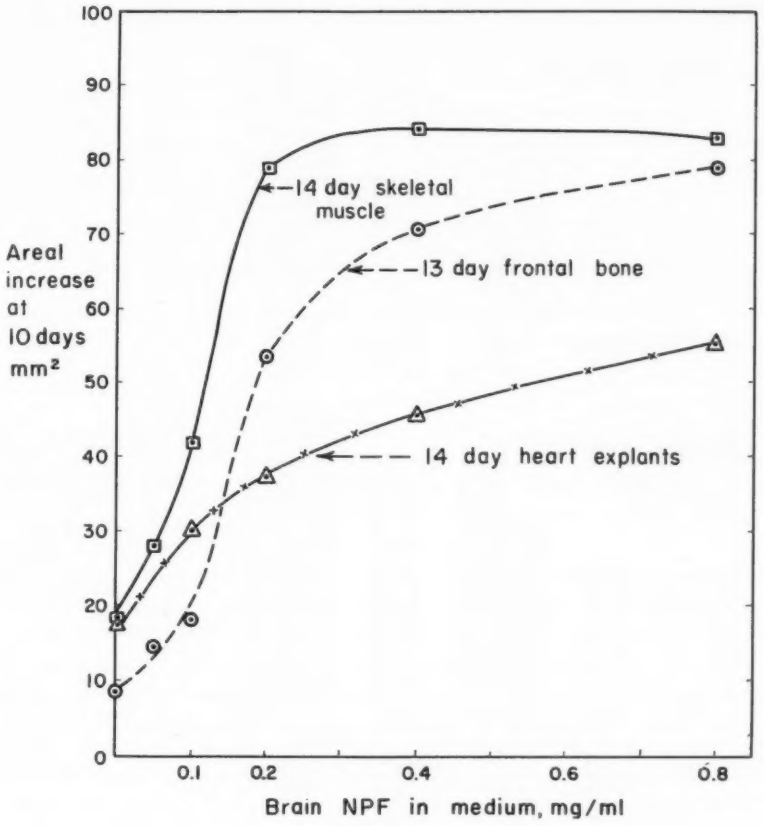

FIGURE 3

Areal increases in cultures of chick fibroblasts supplemented with adult brain NPF.

NP fractions was compared, using a single type of assay culture in any given experiment. The results in terms of areal changes can be expressed graphically by Figure 4. In all cases the addition of NPF led to a net gain in area over unsupplemented controls. The increase was greatest with brain NPF, least with heart NPF and intermediate with NPF from liver or spleen. Relative increases in surface area thus parallel the comparative effect of different NP fractions on the

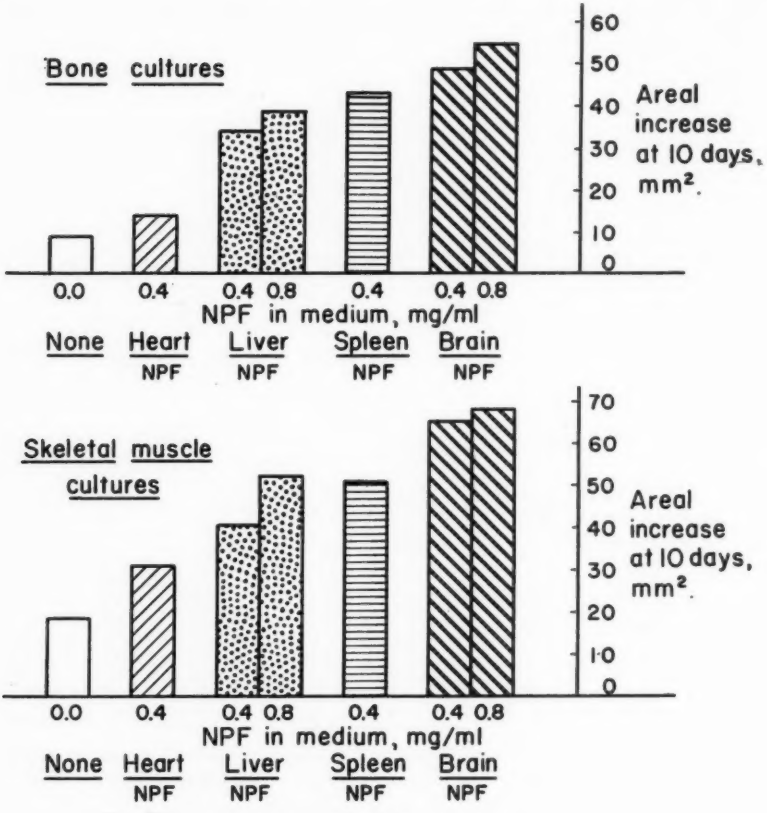

FIGURE 4

Comparative effects of NP fractions from adult chicken organs on surface area of bone and skeletal muscle cultures.

microscopic appearance of assay cultures. The specific activity of various NP fractions thus appears to be brain > liver, spleen > heart. It may be emphasized that the low specific activity of heart NPF was characteristic of assays with heart fibroblasts as well as with the cultures of bone and skeletal muscle shown in Figure 4. No indications were seen of an organ specificity in the action of heart extracts.

Relative activity of nucleoprotein fractions obtained from donors of different age

The preceding experiments show that nucleoprotein fractions which stimulate growth may be obtained from the tissues and organs of adult animals well beyond the cessation of body growth, and even in tissues in which proliferation does not occur except in embryonic stages (brain, heart). It is interesting to know, however, whether NP fractions stimulate growth more effectively if obtained from tissues in active growth, and whether the total yield per unit mass is greater in the latter case. We have accordingly prepared NP fractions from brain, liver and heart of newly hatched (one day) chicks, and have compared the effects of both adult and one-day organ fractions with the NPF prepared from entire 12-day embryos.

The relative yields of NP fractions prepared from these various sources have been tabulated in Table 1 (p. 56). These data suggest that the level of NPF per unit wet weight is essentially the same from embryo to adult. Cessation of growth in adult organs, therefore, does not stem from a reduction in amount of the tissue growth factors under study here.

The relative activity of NP fractions from one-day chicks was determined through a series of experiments which closely paralleled the investigations previously described with extracts from adult organs. Using a single NP fraction (brain) response curves were obtained with cultures of bone, skeletal muscle, and heart. The results are shown in Figure 5, which may be compared with Figure 3 for adult brain NPF. The identity of these two groups of data is striking and extends even to the detailed form of individual curves. These data show clearly that the specific activity of brain NPF does not change from the time of hatching to the adult stage. Similarly Figure 6 contrasts the activity of NP fractions obtained from different organs of one-day chicks and can be compared to Figure 4 for adult organs. The order of specific activity for individual NP fractions is the same in both cases. It seems apparent, therefore, that there are no significant differences in specific activity or total amount of NP fractions from the juvenile and adult organs under study.

Assays were also performed with embryonic NPF. The results showed activity in cultures of heart, bone and skeletal muscle as found with adult and juvenile NP fractions. Some of the data ob-

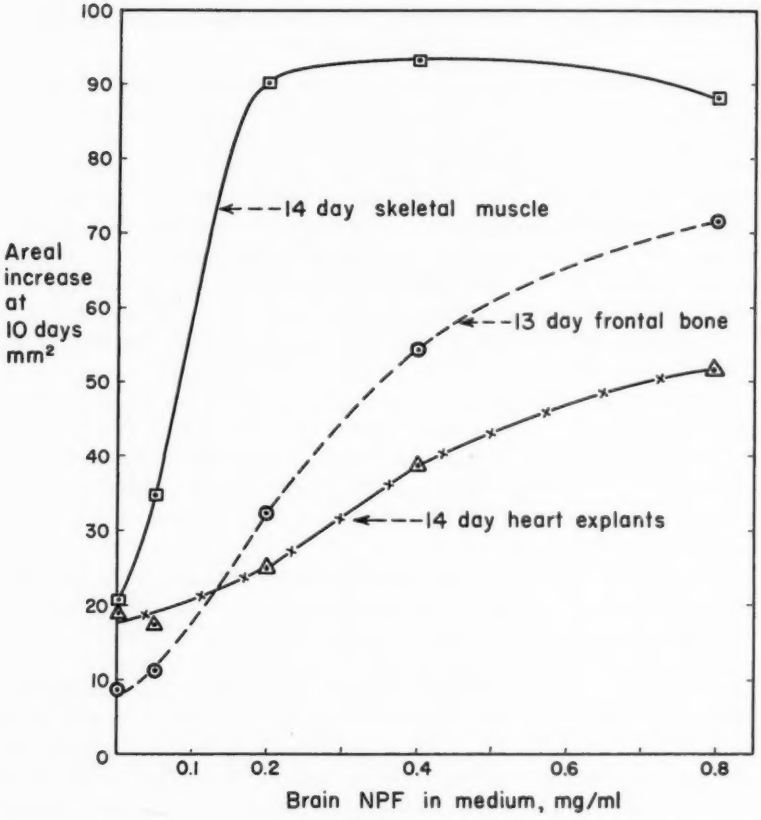

FIGURE 5

Areal changes in cultures of chick fibroblasts supplemented with brain NPF from one-day chicks.

tained are tabulated in Table 3, together with comparable data for brain NPF, the most active fraction, from one day and adult organs. The figures show that the specific activity of NPF from embryonic sources does not exceed, and is in fact slightly less than that of brain NPF from adult or juvenile organs. This finding further underlines the essential similarity in occurrence and action of NP fractions in tissue culture, whether the NP fractions originate in actively grow-

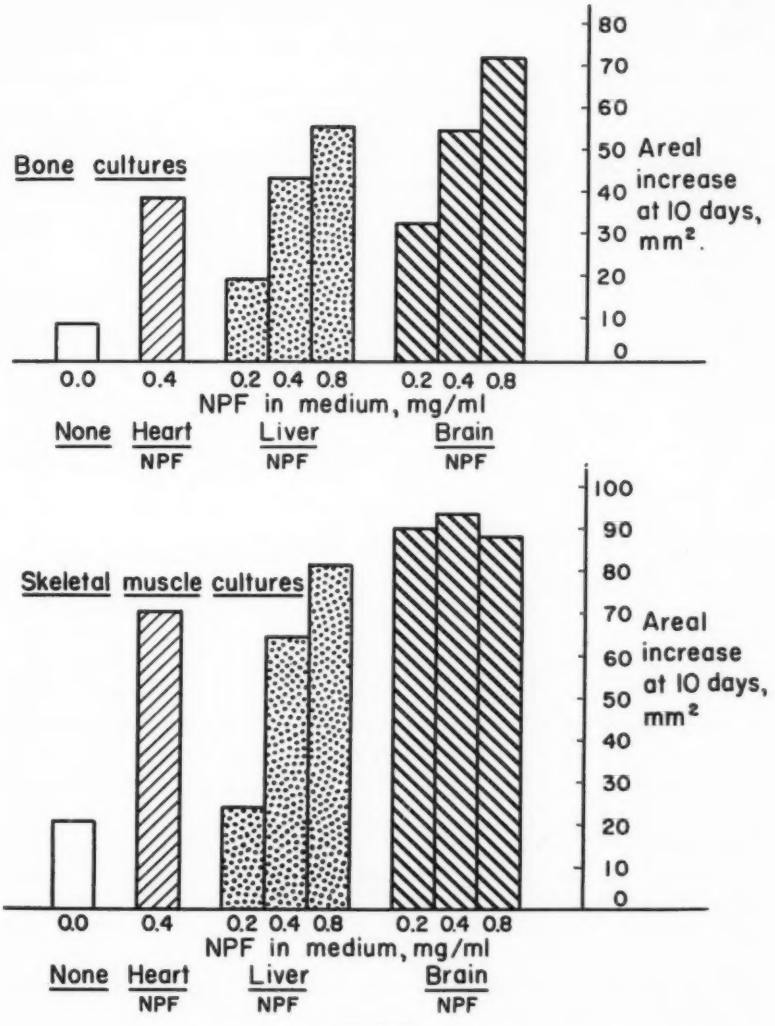

FIGURE 6

Relative activity of NP fractions from organs of one-day chicks in cultures of chick fibroblasts.

ing embryos, or from adult chickens far along the plateau stage in body size.

TABLE 3
Comparison of Areal Increase and Glycolysis in Cultures of Chick Fibroblasts Supplemented with NPF from Donors of Different Age.

Source of NPF Donor	Organ	Type of assay culture	Concentration of added NPF, mg./ml.	Areal increase* at 10 days, mm.^{2}	Lactic acid** at 7 days, µgm./ml.
Adult (2-3yrs.) chickens	Brain	Frontal bone	none	8.8 ± 0.4	182
			0.4	70.8 ± 3.5	605
			0.8	79.1 ± 1.3	675
		Skeletal muscle	none	18.4 ± 2.4	370
			0.4	84.2 ± 6.5	579
			0.8	83.0 ± 2.2	618
one day chicks	Brain	Frontal bone	none	8.7 ± 0.6	195
			0.4	54.4 ± 3.2	550
			0.8	71.8 ± 1.1	630
		Skeletal muscle	none	20.8 ± 2.8	283
			0.4	93.4 ± 7.6	497
			0.8	88.4 ± 8.8	612
12 day chick embryos (whole embryo)		Frontal bone	none	8.0 ± 1.2	127
			0.4	41.5 ± 2.4	330
			0.8	53.8 ± 3.9	490
		Skeletal muscle	none	11.0 ± 1.0	280
			0.4	30.0 ± 2.5	437
			0.8	49.0 ± 2.7	722

* Mean value and standard error (Snedecor, 1946). Six cultures in each group.
** Duplicate determinations on pooled supernatants from six cultures in each series.

Comparative effects of streptomycin and acetone fractions from adult organs

In the preceding sections we have detailed the growth-promoting properties of nucleoprotein fractions obtained by precipitation with streptomycin. The question arose whether these NP fractions were comparable to the active preparations obtained from adult organs by Margoliash and Doljanski (1950) by precipitation with acetone. For purposes of comparison, therefore, acetone-insoluble (AI) fractions were prepared from organs of adult chickens, using the methods of Margoliash as described in a previous section (p. 55). The principal assays of these materials were performed with cultures of frontal bone, although additional data were obtained on the responses of fibroblasts from skeletal muscle and heart respectively.

In general, the effects of AI and NP fractions run parallel in assay cultures: increases in surface area and production of lactic acid (Table 4), radial orientation of cells and changes in colonial pattern

TABLE 4
Effects of Acetone-Insoluble Fractions from Adult Chicken Organs on Plasma Cultures of Chick Frontal Bone.

Source of AI fraction	Concentration in medium, mg/ml	Areal increase* at 10 days, mm^2	Lactic acid** at 7 days, μgm/ml
None	—	10.3 ± 0.7	152
Brain	0.4	38.9 ± 5.0	389
	1.9	105.7 ± 2.2	564
	3.8	140.9 ± 2.4	675
Heart	0.3	33.9 ± 2.5	255
	1.5	75.6 ± 4.8	483
	3.1	96.9 ± 5.7	595
Liver	0.3	38.0 ± 3.2	305
	1.4	71.4 ± 5.1	559
	2.9	49.3 ± 8.6	407
Spleen	0.2	60.7 ± 4.0	547
	1.1	62.9 ± 5.0	541
	2.2	67.9 ± 7.5	569

* Mean value and standard error (Snedecor, 1946). Six cultures in each series.
** Duplicate determinations on pooled supernatants from six cultures in each series.

in the peripheral outgrowth. Cultures with AI supplements, however, were more vacuolated and refractile, and showed more variation in the appearance of cells in the outgrowth zone. These observations may reflect partial denaturation of active proteins in AI fractions as compared to the streptomycin fractions, which were prepared by a milder procedure. It is also possible that the AI fractions contained inhibitors as well as stimulating factors, owing to the nonspecific nature of acetone as a precipitating agent.

The data presented in Table 4 show that AI fractions from adult brain are most active, followed by preparations from adult heart. The response to spleen AI fractions reaches a plateau at low concentrations and shows no further increase at higher levels of the test fraction. AI fractions from liver are inhibiting at high concentrations. A similar pattern of effects was obtained with other assay systems (fibroblasts from heart and skeletal muscle). These results are in essential agreement with the findings of Doljanski and his collaborators (Margoliash and Doljanski, 1950).

The specific activity of AI and NP fractions can be compared in part by examining the data in Table 4 with reference to Table 3 as well as Figures 3 and 4. With the exception of preparations obtained from adult heart, the NP fractions consistently have a higher specific activity than do the corresponding acetone fractions. Brain NPF at 0.4 mg/ml, for example, gives nearly double the response obtained with brain AIF at this level in terms of areal increase or lactic acid production: These results suggest a more selective isolation of active factors by streptomycin treatment as compared to the acetone method.

Discussion

The results of the present experiments are in agreement with the work of Doljanski and his collaborators (Margoliash and Doljanski, 1950) in emphasizing that growth-stimulating agents occur in adult and juvenile organs as well as in embryos. It appears that the active factors include or are associated with substances of high molecular weight, and that these may be obtained by precipitation with either streptomycin or acetone. Of these two methods the streptomycin procedure provides a more selective separation and yields a product of higher specific activity in culture. The acetone fractions contain a much higher amount of heme-like substances, as evidenced by marked peaks in the absorption spectra at 405-408 mμ. In addition the acetone fractions appear to be contaminated to a variable degree with small molecules (e.g., amino acids and co-factors) which show restricted solubility in organic solvents. It may be emphasized that dialyzable factors from chick embryos are themselves growth promoting in assay cultures (Harris, 1953). The presence of similar materials in the acetone fractions thus complicates the study of protein factors or other large molecules. With the streptomycin procedure this difficulty does not arise, since substances of low molecular weight are largely if not entirely removed by the repeated dialysis which this method entails.

The nature of the growth-stimulating agents in the streptomycin precipitates has not yet been ascertained. These fractions whether from adult, juvenile, or embryonic tissues show an ultraviolet absorption spectrum characteristic of ribonucleoproteins. The association of RNA, protein and growth is an attractive one, stemming from a number of other fields of investigation (Brachet, 1950; Pollister, 1954; Gale and Folkes, 1955). In the present experiments, however, no cor-

relation was observed between the percentage of nucleic acid and specific activity of different extracts in plasma cultures. The most active fractions were consistently obtained from brain which shows the lowest content of nucleic acid of the various fractions tested (Table 2). It is possible that the total nucleic acid in the several fractions represents a variable proportion of free and protein-bound RNA, and that the free form is inert. Streptomycin precipitates free nucleic acids present in tissue extracts as well as nucleoproteins (von Euler and Heller, 1948). Electrophoretic studies on embryonic NPF (Kutsky, 1953, 1956) have in fact shown a fast-moving subcomponent of free RNA, which had no activity in subsequent assays. Similarly, a number of recent attempts to demonstrate growth stimulation with high polymer RNA from adult and embryonic tissues have all been negative (Kutsky, 1953 and unpublished experiments). The possibility thus exists that a variable quantity of free nucleic acid as "ballast" in the assay fractions may obscure the correlation between protein-bound nucleic acid and growth.

On the other hand it is equally possible that stimulation of growth by streptomycin precipitates may be associated with protein as such, rather than with specific nucleoproteins. Nucleic acid may play a stabilizing role for these labile tissue proteins under experimental conditions. In this case it should be possible by further modifications of procedure to obtain growth stimulation by tissue proteins alone, essentially free of nucleic acid. This possibility is under active study at the present time.

Acknowledgment

It is a pleasure to acknowledge the technical assistance and material aid in the present investigation of Mrs. Irene Cady, Mr. Richard Underwood, Mr. Vernon Summerfelt, and Mr. Al Geiger.

Summary

1. Nucleoprotein fractions (NPF) were obtained from adult and juvenile chicken organs by precipitation from saline extracts by streptomycin. The resulting preparations are high in RNA and show absorption spectra characteristic of ribonucleoproteins.

2. All NP fractions stimulated active growth when added to p'asma cultures containing fresh explants of chick embryonic tissues. Large increases were obtained in surface area and production of lactic acid

as well as characteristic alterations in cell morphology and colonial pattern.

3. Cultures of chick heart, skeletal muscle, and frontal bone all showed graded responses to increasing levels of NP fractions. No indications were found of organ-specific stimulation.

4. The effects of NP fractions from different organs were generally similar but specific activity followed the order brain > spleen, liver > heart.

5. The total yield and specific activity of NP fractions from individual organs did not differ significantly between juvenile and adult stages. NPF from whole chick embryos fell within the range of activities for adult fractions.

6. Although growth stimulation is associated with nucleoprotein fractions the nature of the active factors has not yet been ascertained. No correlation was found between specific activity and total nucleic acid content of individual fractions.

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